Oral treatments for amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar*

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Declaration

This thesis contains no material which has been accepted for a degree or diploma by the university or any other institution, except by way of background information and duly acknowledged in the thesis and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgements are made in the text of this thesis. At the time of publication, Chapter 2 had been submitted, Chapter 5 was in press and Chapters 3 and 4 had been published within peer reviewed journals.

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Statement of Co-Authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

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Abstract

Neoparamoeba spp. is a marine amphizoic protozoan parasite which infects the gills of marine cultured Atlantic salmon, *Salmo salar*, worldwide causing amoebic gill disease (AGD). Amoebic gill disease is a significant health issue affecting the production of sea-caged Atlantic salmon in Australia with farms experiencing outbreaks regularly throughout the year. It accounts for 10-20% of the gross value of production due not only to the cost of treating and managing the disease, but also to loss of fish condition, increased feed conversion ratio (FCR), lost growth and sometimes mortalities. The current mitigation strategy for AGD is the administration of a freshwater bath to affected sea-caged fish. However, this method is becoming less effective with an apparent increase in bathing frequency over the past few years. The increase in baths has fuelled a rise in already high production costs to the Australian Atlantic salmon industry to approximately 20% annually. This thesis aims to identify an improved method of treatment for AGD either through development of a stand alone in-feed treatment or an in-feed treatment used in conjunction with the current freshwater treatment strategy.

This thesis investigates *in vitro* and *in vivo* effects of bithionol and bithionol sulphoxide on both *Neoparamoeba* spp. and Atlantic salmon. Initially, toxicity to *Neoparamoeba* spp. was examined *in vitro* using isolated gill amoeba and exposing them to seawater, freshwater, alumina (10 mg L^{-1}), bithionol and bithionol sulphoxide at 10, 5, 1, 0.5 and 0.1 mg L^{-1}. The assays were observed for 72 h with viable amoeba counts using trypan blue exclusion conducted at 0, 24, 48 and 72 h. Both bithionol and bithionol sulphoxide were toxic to *Neoparamoeba* spp. *in vitro*

at all concentrations examined. A similar toxicity to freshwater water was observed with bithionol and bithionol sulphoxide at 10 and 5 mg L^{-1} following a 72 h treatment. However, freshwater was the most effective with only 6% viable amoebae seen after 24 h and no viable amoeba observed a further 24 h later.

Once identified as toxic to *Neoparamoeba* spp. *in vitro*, an assessment of the toxicity of bithionol to Atlantic salmon and the efficacy as an AGD treatment was evaluated. This was conducted via a bath treatment to Atlantic salmon and rainbow trout, *Oncorhynchus mykiss*, held in either fresh or seawater using concentrations between 1 and 35 mg L^{-1} to examine toxicity. To examine efficacy, a bath treatment of AGD-affected Atlantic salmon and rainbow trout at 1 to 25 mg L^{-1} was also evaluated. To examine toxicity, fish were bathed for 1, 3 and 6 h in bithionol, an anti-protozoal at 0, 1, 5, 10, 25 and 35 mg L^{-1}, with toxicity determined by time to morbidity and histological examination of internal organs. Efficacy was examined by bathing AGD-affected Atlantic salmon and rainbow trout for 1 h at bithionol concentrations of 1 to 25 mg L^{-1}. Efficacy was determined by examining gill amoeba counts and identifying percent lesioned gill filaments at 1 and 24 h after bath exposure to bithionol. Only bithionol at 1 mg L^{-1} was considered non-toxic with no signs of morbidity. Bithionol appeared to be more toxic in seawater than freshwater, exhibiting a higher rate of morbidity, and had no acute effects on gill Na^+/K^+ ATPase and succinic dehydrogenase, or plasma osmolality and chloride concentration. Bithionol reduced the percentage of lesioned gill filaments to the same level as freshwater.

Bithionol was examined as an in-feed treatment for AGD with and without the administration of a freshwater bath. Bithionol when fed as a two week prophylactic or therapeutic treatment at 25 mg kg^{-1} feed delayed the onset of AGD pathology and reduced the percent lesioned gill filaments. Administration of a 3 h freshwater bath at 28 days post-exposure significantly reduced amoebae numbers to a similar level across all treatments; in contrast gross gill score and percent lesioned filaments were reduced proportionally. Hence, the control was significantly higher than both bithionol treatments. Following the freshwater bath, clinical signs of AGD recurred at a similar level across all treatments although controls clinical signs were significantly higher than the bithionol treatments to begin with. Palatability was not a problem with mean feed intake of bithionol over the trial duration higher compared to both the oil and plain controls.

This thesis has identified that bithionol at 25 mg kg^{-1} feed, when fed as a two week prophylactic or a therapeutic treatment, delayed and reduced the intensity of AGD pathology. Such findings as the identification of bithionol as a possible in-feed treatment for AGD and its effectiveness against numerous other parasites suggests that bithionol could be worth examining in other aquatic animal diseases. Furthermore, bithionol warrants further investigation as a potential in-feed treatment for AGD in Atlantic salmon especially in regards to a combination therapy with the current freshwater mitigation.

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List of Abbreviations

AGD	amoebic gill disease
ANOVA	analysis of variance
AS	Atlantic salmon
ATP	adenosine 5'-triphosphate
BW	body weight
C	control feed
CRC	Cooperative Research Centre
EC_{50}	median effective concentration
EDTA	ethylenediaminetetraacetic acid
FCR	feed conversion ratio
FDA	Food and Drug Administration
FRDC	Fisheries Research and Development Corporation
fw	freshwater
H & E	haematoxylin and eosin
IFAT	indirect fluorescent antibody test
ILU	inter lamellae units
IQR	inter-quartile range
K	Fulton's condition factor
LC_{50}	median lethal concentration
LCEE	L-cysteine ethyl ester
LT	lethal time
LT_{50}	median lethal time
Na	not applicable
NADH	reduced nicotinamide adenine dinucleotide
Na^+/K^+ ATP	sodium potassium adenosine 5'-triphosphate
PB	prophylactic bithionol feed
PCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
PE	post exposure
PLOs	*Perkinsiella amoebae* like organisms
PO_2	partial pressure of oxygen
RBT	rainbow trout
RPR	relative percent reduction
R & D	research and development
SALTAS	Salmon Enterprises of Tasmania Pty Ltd
SDH	succinic dehydrogenase
SEID	sucrose, Na_2EDTA, imidazole and Na^+-deoxycholic acid
SEM	standard error of the mean
SGR	specific growth rate
sw	seawater
TB	therapeutic bithionol feed

Table of Contents

List of Figures

Number *Page*

List of Tables

Number *Page*

CHAPTER 1

GENERAL INTRODUCTION

1 General Introduction

1.1 The impact of amoebic gill disease (AGD) on Atlantic salmon, *Salmo salar*, in Australia

Amoebic gill disease is a significant health issue affecting the production of sea-caged salmon in Tasmania, Australia, with farms experiencing regular outbreaks throughout the year (Clark and Nowak, 1999; Munday *et al.*, 2001; Nowak *et al.*, 2002) accounting for 10-20% of the gross value of production (Munday *et al.*, 2001). However, AGD outbreaks are generally most severe during the summer months; previous studies have shown that salinity and temperature are significant environmental factors influencing the outbreaks of AGD (Clark and Nowak, 1999; Douglas-Helders *et al.*, 2001b; Adams and Nowak, 2003). Other factors that are thought to influence AGD outbreaks include rainfall (Munday *et al.*, 1993; Clark and Nowak, 1999; Nowak, 2001), dissolved oxygen (Clark and Nowak, 1999), biofouling on cages (Tan *et al.*, 2002), husbandry techniques (Clark and Nowak, 1999), removal of mortalities (Douglas-Helders *et al.*, 2000), the general health status of the fish (Nowak, 2001) and possibly bacterial gill populations (Bowman and Nowak, 2004; Embar-Gopinath *et al.*, 2005).

The increase gross production costs mentioned above is due not only to the cost of treating and managing the disease, but also to loss of fish condition, increased feed conversion ratio (FCR) and lost growth attributed to an increase in standard metabolic rate (Leef *et al.*, 2007a). If left untreated fish mortalities can reach over 50% (Munday *et al.*, 1990) and furthermore pose a greater financial loss to the

industry. As well as mortalities, reduced growth can also be an issue taking a longer time to reach market size (Rodger and McArdle, 1996; Dyková *et al.*, 1998).

1.2 The etiology of amoebic gill disease

1.2.1 The agents associated with amoebic gill disease

The presumptive causative agent of AGD is a marine amphizoic protozoan parasite, identified as *Paramoeba pemaquidensis* (Page, 1970) and then reclassified to *Neoparamoeba pemaquidensis* (Page, 1987). More recently other amoeba species, *N. branchiphila* and *N. aestuarina*, were cultured from AGD affected fish, but it was not known if one or all of these species were the aetiological agents of AGD (Dyková *et al.*, 2005). Subsequently, a new amoeba species designated *Neoparamoeba perurans* n. sp. has been identified as the predominate aetiological agent of AGD affecting Atlantic salmon, *Salmo salar*, culture in Tasmania invalidating previous classifications (Young *et al.*, 2007).

Neoparamoeba (Page, 1987) are small, naked and lobose amoebae that form dactylopodiate subpseudopodia in their locomotive state and belong to the family Vexilliferidae. Some defining characteristics enabling classification of this genus include the fact that forms lack the well organised cell-surface structures of other vexilliferids including surface scales and hexagonal glycostyles. They also possess a nucleus plus one or more 'parasomes'. These 'parasomes' are described as *Perkinsiella amoebae* like organisms (PLOs) and are eukaryotic endosymbionts, closely related to the kinetoplastid *Ichthyobodo* (Dyková *et al.*, 2003).

Neoparamoeba spp. is not confined to Australian waters, with AGD outbreaks worldwide including Ireland, Spain, New Zealand, USA (Washington State and California), Chile, and more recently Norway (reviewed by Munday *et al.*, 2001; Nowak *et al.*, 2002; Steinum *et al.*, 2008). Amoebic gill disease has also been found to affect a number of other species including Atlantic salmon (Kent *et al.*, 1988; Munday *et al.*, 1990), rainbow trout, *Oncorhynchus mykiss*, (Munday *et al.*, 1990), brown trout, *S. Trutta*, (Munday *et al.*, 2001), coho salmon, *O. Kisutch*, (Kent *et al.*, 1988), chinook salmon, *O. Tshawytscha*, (Kent *et al.*, 1988), turbot, *Scophthalmus maximus*, sharpsnout seabream, *Diplodus puntazzo*, and European sea bass, *Dicentrarchus labrax*, (Dyková *et al.*, 2000; Dyková and Novoa, 2001). Atlantic salmon are reported to be the most susceptible of cultured species (Munday *et al.*, 2001), but interestingly AGD has not been reported in other wild fish species including red cod, *Pseudophycis bachus*, sand flathead, *Platycephalus bassensis*, and jack mackerel, *Trachurus declivus*, even when present around farms with severely affected salmon (Nowak *et al.*, 2004).

Throughout this thesis, the causative agent of AGD is referred to as *Neoparamoeba* spp. Furthermore, this work was conducted prior to the discovery of *N. perurans* and *Neoparamoeba* spp. is used in reference to the aetiological agent of AGD. However, irrespective of the term used to describe the organism, it is referring to the isolated gill amoebae and hence the species of *Neoparamoeba* which are the aetiological agents of AGD.

1.2.2 Morphology and identification

When freshly isolated from the gills of infected fish, *Neoparamoeba* spp. appear in their free floating form as spherical and possess multiple pseudopodia (Kent *et al.*, 1988; Munday *et al.*, 1990; Rodger and McArdle, 1996; Dyková *et al.*, 1998). However, a more lobose form is assumed when *Neoparamoeba* spp. are attached to a substrate with the nucleus and "parasome(s)" generally visible (Fig 1.2-1). In histology sections using either wax or resin, both the nucleus and "parasome(s)" are visible and the trophozoites often appear highly vacuolated (Roubal *et al.*, 1989; Munday *et al.*, 1990; Dyková *et al.*, 1995). While morphological characteristics distinguish *Neoparamoeba* from other vexilliferids, attempts to distinguish members of the genus *Neoparamoeba* using morphological characteristics alone have been unsuccessful (Dyková *et al.*, 2000; Dyková *et al.*, 2005).

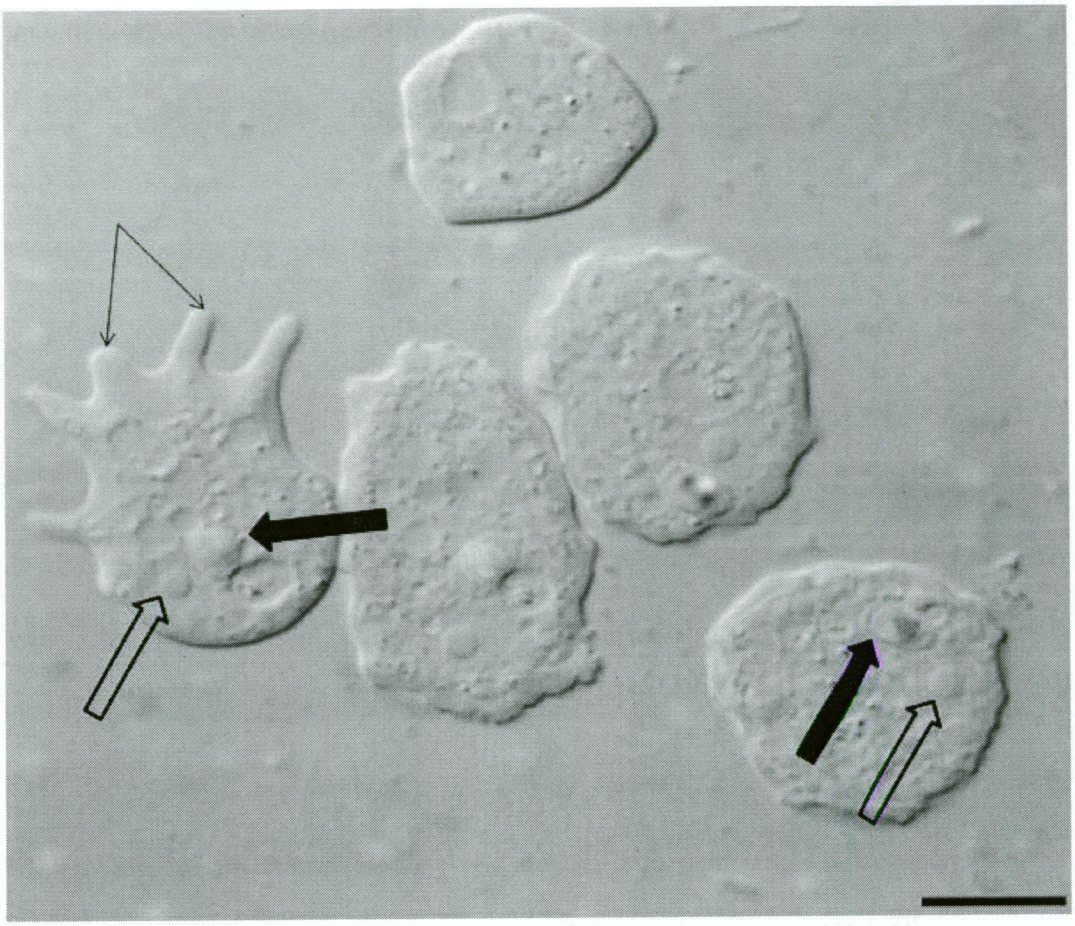

Fig 1.2-1. Attached trophozoites of *Neoparamoeba pemaquidensis* from a sediment isolate showing pseudopodia (fine arrow), nucleus (unfilled arrow), and "parasome" (filled arrow) (plate courtesy of Dr Bret Robinson) Bar = 20μm.

Detection of *Neoparamoeba* spp. can be achieved using both pathogen specific and non-specific tests with samples being obtained either lethally or non-lethally to the fish. A wet mount preparation, whereby gill mucus is smeared onto a glass slide, dried, stained and examined microscopically, is based primarily on the morphology of the pathogen. Histology is reliable but the obtaining of histology samples is lethal to the fish and once again reliant upon morphology. Specific stains, such as indirect fluorescent antibody test (IFAT) (Howard and Carson, 1993), immuno-cytochemistry (Zilberg and Munday, 2000; Howard, 2001) and immune-dot blot (Douglas-Helders *et al.*, 2001a), can also be used on histological sections and gill smears, although not particularly reliable for assessing infections on some farms or assessing treatment efficacy (Harris *et al.*, 2004; Harris *et al.*, 2005). Immunological detection of *Neoparamoeba* using anti-*N. pemaquidensis* antiserum was reported successful in identifying different *Neoparamoeba* spp. (Douglas-Helders *et al.*, 2001a). However, the anti-*N. pemaquidensis* antiserum was later shown to bind non-specifically to other marine amoebae (Morrison *et al.*, 2005). Recently, *N. branchiphila* was characterised using a combination of morphological and molecular phylogenetic analyses inferred from 18S rRNA gene sequences (Fiala and Dyková, 2003; Dyková *et al.*, 2005) and was clearly differentiated from *N. pemaquidensis* and *N. aestuarina*. Furthermore, this resolved inter-specific relationships within the *Neoparamoeba* group (Fiala and Dyková, 2003; Dyková *et al.*, 2005). Hence, species-specific diagnostic tools were developed, based upon 18S rRNA gene amplification by polymerase chain reaction (PCR) to study disease aetiology where *Neoparamoeba* spp. were the presumptive pathogens (Elliott *et al.*,

2001; Fiala and Dyková, 2003; Wong *et al.*, 2004; Dyková *et al.*, 2005; Mullen *et al.*, 2005; Young *et al.*, 2007).

1.3 Pathology and pathophysiology of amoebic gill disease in Atlantic salmon

Amoebic gill disease is characterised clinically or macroscopically by the presence of gross gill lesions which are characterised by focal or multifocal, raised white mucoid patches, profuse mucus production and mucous cell proliferation (Clark and Nowak, 1999; Adams and Nowak, 2001; Roberts and Powell, 2003b). Gross examination of gills for the presence of white patches is routinely used by the Tasmanian Atlantic salmon industry for gross tentative diagnosis of AGD and determination of treatment times. Other clinical signs reported for AGD include lethargy, loss of appetite and respiratory distress manifested as rising to the water surface and an increased ventilation frequency; however, due to routine farm monitoring and early treatment, disease rarely progresses to the point at which such behaviour is elicited (Munday *et al.*, 1990; Munday *et al.*, 2001). The prominent microscopic feature of AGD is multi-focal hyperplasia of the lamellar epithelium which, in turn, results in the fusion of secondary lamellae and formation of interlamellar vesicles, which coincides with a reduction in chloride cell number and an increase in mucous cells (Dyková *et al.*, 1995; Powell *et al.*, 2001; Adams and Nowak, 2003; Roberts and Powell, 2003b). Typically AGD lesions are associated with *Neoparamoeba* spp. present within the vicinity of the hyperplastic tissue (Nowak and Munday, 1994). As the disease progresses, the hyperplastic tissue along with associated amoeba are sloughed off, which has been thought to be a

possible 'self-cleaning' action important for the recovery from the disease (Munday *et al.*, 2001).

Physiologically, Munday *et al.* (1990) reported elevated blood sodium levels in Atlantic salmon severely affected with AGD; however, Powell *et al.* (2001) reported that there were no clinical observations regarding this. Fish affected by AGD are known to have lower blood oxygen partial pressure (PO_2) despite the fact that oxygen uptake rates are not affected under normoxic conditions in clinically affected fish (Powell *et al.*, 2000). Elevated blood carbon dioxide (tensions) (PCO_2) are also seen in AGD-affected Atlantic salmon resulting in respiratory acidosis (Powell *et al.*, 2000; Leef *et al.*, 2005b). It has been suggested that this respiratory acidosis exhibited in AGD-affected fish may be a result of the presence of AGD lesions and/or an increase in branchial mucous secretion which in turn leads to a reduced gill surface area and diffusive conductance (Powell *et al.*, 2000). Even though it has been shown that AGD-affected fish are acidotic, the presence of AGD did not appear to contribute to respiratory failure when AGD-affected fish were exposed to hypoxic condition at approximately 25% saturation. Furthermore, suggesting that although physiological mechanisms including increased blood flow and perfusion within the gill, AGD-affected fish were able to maintain oxygen transport (Powell *et al.*, 2000).

Amoebic gill disease is associated with a chronic vascular hypertension as well as compensatory cardiac remodelling. Whilst examining the cardiovascular effects of AGD, Powell *et al.* (2002b) hypothesised that in cases of chronic AGD, a

compensatory response for high ventral (afterload) and dorsal (preload) aortic pressure (as seen by Powell *et al.* (2002a)) may be to promote morphological changes in both ventricle length and the thickening of ventricular compact muscle. Atlantic salmon affected with AGD have been shown to have high systemic resistance and lowered cardiac output when compared to naïve counterparts, a characteristic of Atlantic salmon not seen in either rainbow or brown trout (Leef *et al.*, 2005a). There is evidence to suggest that AGD may possibly be associated with cardiovascular dysfunction (Powell *et al.*, 2002a; Powell *et al.*, 2002b); this was supported by Leef *et al.* (2005a; 2007b).

When affected with AGD, the critical swimming speed and subsequent recovery of Atlantic salmon is significantly affected. Furthermore, following a freshwater bath the swimming performance was found to increase with the suggestion that this post-bath increase observed is due to the removal of amoeba and hyperplastic gill tissue (M. Jones pers. comm.). The excess post-exercise oxygen consumption doubled in AGD-affected fish compared to uninfected controls, hence infected fish need longer to repay their oxygen debt and recover oxygen consumption rates to routine levels (M. Jones pers. comm.). This supports earlier observations of Powell *et al.* (2002a) where freshwater bathing also led to a decrease in dorsal aortic pressure in clinically affected fish. Oxygen consumptions rates have been used indirectly to measure metabolic rate in AGD-affected Atlantic salmon. It was reported that both routine metabolic rate and metabolic scope are significantly affected, with the magnitude of effect linked to the severity of AGD. Suggesting

that AGD infection does have a significant metabolic cost associated however, only at high infection levels (M. Jones pers. comm.).

1.4 Freshwater therapy for amoebic gill disease

1.4.1 History

The mitigation of AGD in Tasmania, Australia, is mostly due to freshwater bathing for 2-4 h, which was first recommended by Foster and Percival (1988). On the farm, fish are routinely sampled non-lethally to examine the presentation of raised white mucoid patches, and given a gill score based upon the presence of patches, mucus and colouration of the gills (Table 1.4-1) (Powell *et al.*, 2001; Fisk *et al.*, 2002). Following gross diagnosis, fish are bathed for 2-4 h if required (Parsons *et al.*, 2001a). It is suspected that a combination of osmotic challenge to amoeba, removal of seawater stable gill mucus and the dissolution of gill lesions contributes to treatment success (Munday *et al.*, 2001; Parsons *et al.*, 2001a; Roberts and Powell, 2003b; Adams and Nowak, 2004a). However, the efficacy is variable and has notably become increasingly less effective (Parsons *et al.*, 2001a; Powell and Clark, 2003), possibly due to differing water chemistries (Parsons *et al.*, 2001b). This was supported both *in vitro* and *in vivo* with survival of gill amoeba reported as lower in soft freshwater (low total hardness) compared to hard freshwater (high total hardness) (Powell and Clark, 2003; Roberts and Powell, 2003a). When freshwater bathing was first introduced in the late 1980s, two to three baths were sufficient in providing alleviation from AGD during the marine production cycle (Foster and Percival, 1988; Clark and Nowak, 1999). Presently, however, fish may require up to ten baths in the same period to achieve sufficient

alleviation of AGD (Mitchell, 2001). It has been demonstrated that there was survival of amoebae within mucous samples following bathing and amoebae have been observed within inter-lamellar vesicles suggesting a potential source of recurrent infection (Parsons *et al.*, 2001a). However, this was later refuted by Adams and Nowak (2001) identifying the vesicles as closed structures containing dead or dying amoeba. Furthermore, Clark *et al.* (2003) reported gill associated amoeba numbers return to pre-bath levels ten days following treatment.

Table 1.4-1. Huon Aquaculture Company Dover, Australia method for AGD gross gill lesion scoring scheme.

Infection level	Score	Gross signs
Clear	0	Gills appear clean, healthy and red in colour
Very Light	1	1 mucoid patch, light mucus accumulations
Light	2	2-3 mucoid patches, some paling colour
Medium	3	Established thickened mucoid patches and mucus
Heavy	4	> 3 mucoid patches or a single large patch resulting from patch accumulation

1.4.2 Effects of freshwater bathing

The two major events that occur during commercial freshwater bath treatment include: 1) removal of attached and/or associated trophozoites from the gills, and 2) the osmotic killing of amoebae flushed from the gills to the treatment medium. It was hypothesised that freshwater sloughs mucus and, to some degree, the epithelium from the gills, thus removing amoebae (Munday *et al.*, 2001). Nowak *et al.* (2007) hypothesised that it is likely that amoebae "let go" of a given substrate as

trophozoites succumb rapidly to the osmotic effects of freshwater through swelling or "balling up" and disruption to cytoplasmic organelles.

A number of studies have shown that freshwater bathing has therapeutic effects of promoting repair of the amoeba-induced damage to the gills via the sloughing off of hyperplastic tissue, reducing the number of gill lesions (Munday *et al.*, 2001; Roberts and Powell, 2003a) and the hydration and removal of mucus from the gills (Roberts and Powell, 2003b). The ability of a bath to flush amoebae from the gills into the surrounding treatment medium is most likely to be dependent upon many factors such as: water chemistry, bath duration, velocity of the water and the severity of infection, as well as others not yet considered. The fact that re-infection under experimental conditions can occur after a 3 h freshwater bath treatment without further addition of infective material post-bath suggests amoebae are able to survive within the gill environment during treatment and reproduce disease following return to full salinity (Gross *et al.*, 2004).

During a short-term exposure to freshwater, as is experienced in commercial freshwater bathing, there appear to be no adverse physiological effects on AGD-affected Atlantic salmon and no effects on plasma ions or branchial chloride cells (Powell *et al.*, 2001). However, the number of branchial mucous cells increased coinciding with a change in their histochemical staining and the gill mitochondrial marker succinic dehydrogenase (SDH) activity significantly decreased, suggesting that freshwater bathing has the potential to reduce hyper-ionregulatory capacity of AGD-affected marine Atlantic salmon (Powell *et al.*, 2001). Thus, it was concluded

that when used as a treatment for AGD, a minimum 2 h freshwater bath poses little side effects, neither positive or negative, with regard to the physiological status of the salmon (Powell *et al.*, 2001)

1.5 Alternative treatments for amoebic gill disease

1.5.1 Chemotherapeutant drugs tested to date

A large variety of antimicrobials, disinfectants and detergents have been examined both *in vitro* on isolated gill amoebae and *in vivo* as both bath and feed additives for AGD mitigation. The chemicals that have been examined *in vitro* for amoebicidal or amoebistatic properties include quinacrine, pyrimethamine, levamisole, quinoline, hydroxyquinoline, narasin, napthoquinone, hydrogen peroxide, chlorine dioxide, chloramine-T, L-cysteine ethyl ester, Citrox™, amprolium (Amprolium200™), toltrazuril (Baycox™), bithionol, albendazole (Alben™), caprylic acid, monensin, salinomycin (Bio-Cox™), lasalocid acid (Bovatec™), maduramycin (Cygro™), metronidazole, and bronopol (Pyceze™) (Table 1.5-1) (Alexander, 1991; Howard and Carson, 1993; Powell *et al.*, 2003; Powell and Clark, 2003; 2004; Powell *et al.*, 2005; Powell *et al.*, 2008). Compounds examined as feed and bath chemical additives *in vivo* have generally been identified as ineffective in relieving clinical signs of AGD at the concentrations used (Table 1.5-1). Narasin when fed at 50-60 mg kg^{-1} body weight (BW) for 7 days was found to reduce AGD gill lesions; however, palatability problems were identified and trials were discontinued (Cameron, 1992). Levamisole has been previously examined with varying results; it was found to be ineffective when fed every third day for 15 days at 15 mg kg^{-1} BW (Cameron, 1992). In contrast, 1.25 to 5 ppm

levamisole in freshwater for 2-3 h was reported effective as it significantly augmented the efficacy of the freshwater bath (Findlay *et al.*, 2000). Furthermore, when levamisole was provided as a bath supplement under field conditions, Clark and Nowak (1999) were unable to detect a beneficial effect.

Another chemotherapeutant that has been previously examined with mixed results is chloramine-T. Although listed as ineffective by Munday *et al.* (2001), subsequent studies using chloramine-T as a bath additive have produced some promising yet varied results. Chloramine-T has been found to significantly reduce gill amoeba numbers when added to freshwater during a commercial bathing trial on one farm but not on another and its efficacy depends on factors such as water chemistry (Powell and Clark, 2004). Recently, chloramine-T as an additive to seawater has been shown to be an effective AGD treatment under experimental conditions (Harris *et al.*, 2004; Harris *et al.*, 2005) although it was found to be more acutely toxic to Atlantic salmon in seawater as opposed to freshwater (Powell and Harris, 2004).

Table 1.5-1. Compounds examined as possible treatmzents for amoebic gill disease.

Compound	Type of drug	Type of test	Type of treatment	Relative efficacy	Relative toxicity*	Reference†
Amprolium	Antiprotozoal	*In vitro*		N/A	1	1
Toltrazuril	Antiprotozoal	*In vitro*		N/A	1	1
Albendazole	Antiprotozoal	*In vitro*		N/A	1	1
Caprylic acid	Anti-fungal	*In vitro*		N/A	1	1
Bronopol	Biocide	*In vitro*		N/A	1	2
Formalin	Biocide	*In vitro*		N/A	1	2
Monensin	Ionophore	*In vitro*		N/A	1	2
Salinomycin	Ionophore	*In vitro*		N/A	2	3
		In vivo	In-feed	Low	1	3
Lasalocid acid	Ionophore	*In vitro*		N/A	1	3
		In vivo	In-feed	Low	1	3
Maduramycin	Ionophore	*In vitro*		N/A	1	3
Metronidazole	Metabolic inhibitor	*In vitro*		N/A	1	2
Bithionol	Antiprotozoal	*In vivo*	Bath	Moderate	2	4
Bithionol	Antiprotozoal	*In vivo*	In-feed	Moderate	1	5
Chloramine-T	Disinfectant	*In vivo*	Bath	Moderate	1	6 and 7
Chlorine Dioxide	Disinfectant	*In vivo*	Bath	Moderate	Variable	7
Hydrogen Peroxide	Disinfectant	*In vivo*	Bath	Moderate	2	7
Levamisole	Immunostimulant	*In vivo*	Bath	Moderate	1	8 and 9
L-cysteine ethyl ester	Mucolytic	*In vivo*	In-feed	Moderate	N/A	10

* *In vitro* toxicity to amoebae, *in* vivo toxicity to fish 1 = low to moderate toxicity; 2 = high toxicity

† 1 = (Powell *et al.*, 2003); 2 = (Powell *et al.*, 2005); 3 = (Powell *et al.*, 2008); 4 = (Florent *et al.*, 2007a); 5 = (Florent *et al.*, 2007b); 6 = (Harris *et al.*, 2004); 7 = (Powell and Clark, 2004); 8 = (Findlay *et al.*, 2000); 9 = (Munday and Zilberg, 2003); 10 = (Roberts and Powell, 2005)

1.5.2 Bithionol and bithionol sulphoxide as novel treatments

Bithionol is a white crystalline powder with a faint phenolic odour. It is insoluble in water and has the chemical structure as seen in Fig 1.5-1 (Enzie and Colglazier, 1960; Yang and Lin, 1967). It was examined in 1960 as a treatment for paragonimiasis (lung fluke disease) in humans (Yang and Lin, 1967). In 1962, the discovery of bithionol sulphoxide (Fig 1.5-2) created another opportunity for disease treatment using a compound with allegedly less toxicity than bithionol (Yang and Lin, 1967). There appears to be some conflict in the literature with respect to bithionol and bithionol sulphoxide and how they relate to each other. Durbize *et al.* (2003) suggested that bithionol sulphoxide is a photoproduct of bithionol, whereas Mourot *et al.* (1987) hypothesised that in cows bithionol is a metabolite of bithionol sulphoxide via the reduction pathway. Similarly Meshi *et al.* (1970) found that in rats bithionol sulphoxide was oxidized to both bithionol sulphone and bithionol. Furthermore, they identified that the metabolic fate of these compounds was somewhat different. Bithionol sulphone was excreted mainly in urine as 3,5-dichioro-2-hydroxyphenylsulfonic acid, whereas bithionol was excreted mainly in bile as a glucuronide conjugate.

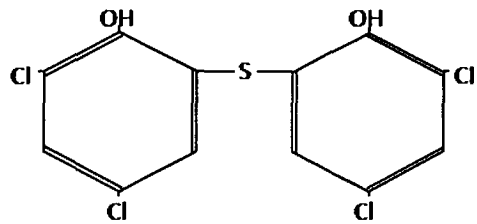

Fig 1.5-1. Chemical structure of bithionol (2,2'-thiobis (4,6-dichlorophenol)).

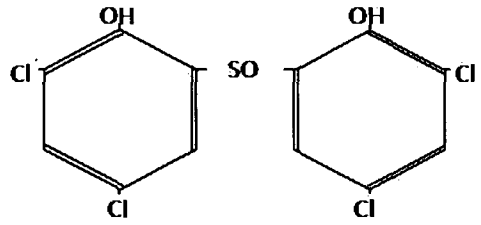

Fig 1.5-2. Chemical structure of bithionol sulphoxide (Bis (2-hydroxy-3,5-dichlorophenyl) sulphoxide).

Bithionol and bithionol sulphoxide have been used as a successful treatment for numerous human disease including paragonimiasis and fascioliasis (Yang and Lin, 1967; Bacq *et al.*, 1991). However, bithionol sulphoxide was found to have stronger anthelminthic activity than bithionol when examined in rats (Meshi *et al.*, 1970). Bithionol and bithionol sulphoxide have also been examined as possible treatments for natural rumen fluke infections in cattle and tapeworm infections in cats, dogs, sheep and chickens (Enzie and Colglazier, 1960; Prasittirat *et al.*, 1997). Bithionol was identified as toxic to medaka, *Oryzias latipes*, with a 96 h LC_{50} of 0.24 mg L^{-1}, whilst the cladoceran, *Daphnia magna*, and the rotifer, *Brachionus calyciflorus*, were both found to be susceptible to bithionol with 48 h and 24 h EC_{50} values of 0.3 and 0.063 mg L^{-1} respectively (Yoshimura and Endoh, 2005).

Bithionol and bithionol sulphoxide have been examined with mixed results as both bath and oral treatments for numerous fish parasites including the flagellates, *Ichthyobodo necator* and *Hexamita salmonis* (Tojo *et al.*, 1994a; Tojo and Santamarina, 1998a), the mongeneans, *Microcotyle sebastis* (Kim and Choi, 1998),

Gyrodactylus spp. (Tojo *et al.*, 1993) and *Pseudodactylogyrus* spp. (Buchmann *et al.*, 1992), and the protozoan, *Trichodina jadranica* (Madsen *et al.*, 2000). Santamarina *et al.* (1991) observed limited toxicity and complete *in vitro* efficacy against *Gyrodactylus* sp. in rainbow trout at 12.5 mg L^{-1}, with a minimum 20 mg L^{-1} reported as efficacious *in vivo*. Tojo *et al.* (1994b) stated that bithionol was efficacious *in vivo* against *I. necator* in rainbow trout at 25 mg L^{-1} for a 3 h bath on two consecutive days; however, higher concentrations exhibited some mortality. Finally, Madsen *et al.* (2000) determined that bithionol at 0.1 mg L^{-1} was an effective treatment against trichodiniasis in European eels, *Anguilla Anguilla*; they found that bithionol had a relatively narrow therapeutic index. Bithionol has also been examined for its toxicity to *Neoparamoeba* spp. *in vitro* and was found to be amoebicidal at 1 and 10 mg L^{-1} (Powell *et al.*, 2003); however, when examined *in vivo* there were palatability issues (Powell unpublished).

Bithionol at 40 g kg^{-1} feed was offered for 10 days at 2% BW per day to rainbow trout infected with *Spironucleus salmonis* formely known as (*H. salmonis*), *Gyrodactylus* sp. or *I. necator* and resulted in a reduction in parasite load. Bithionol eliminated approximately 80% of *S. salmonis* from rainbow trout while both *Gyrodactylus* sp. and *I. necator* infections were reduced from a high to low intensity (Tojo and Santamarina, 1998a; b; c). Kim and Choi (1998) reported bithionol administered in feed at 100–200 mg kg^{-1} BW significantly reduced the number of monogeneans, *M. Sebastis*, on the gills of cultured rock fish, *Sebastes schlegeli*, with a 20 day feeding duration being most effective.

1.6 Fish health management - the host, pathogen and environment interaction

In common with mammalian and poultry farming, the aquaculture industry is subject to a wide range of diseases; in fact Hedrick (1998) describes disease as an integral part of the existence of all animals. Some of these diseases can be controlled. However aquaculture involves intensive animal husbandry, maintaining large numbers of animals in a relatively limited space; it is these large numbers and limited space which has led to the exacerbation of diseases and an increased risk of disease outbreaks (Stoffregen *et al.*, 1996). In contrast to mammalian therapeutics, the use of pharmaceutical substances, in particular antiparasitic drugs, in fishes is limited (Athanassopoulou *et al.*, 2004). It is restricted to the use of anaesthetic agents and anti-infective agents for parasitic and microbial diseases (Burka *et al.*, 1997).

Veterinarians, fish biologists, and ecologists differ significantly in their approach to infectious disease. Veterinary and human medicine believes that for a particular disease, Koch's postulates must be satisfied (Hill, 1965; Evans, 1976). However, in aquaculture the pathogenicity of infectious agents may be so severe that infected fishes die before they can be detected; consequently satisfying Koch's postulates may not be possible. This does not, however, mean that their potential to cause disease should be ignored (Bakke and Harris, 1998).

Disease covers a wide spectrum from acute mortality to benign syndromes; however, they all display a deviation from the normal structure or function of the

host (Hedrick, 1998). Generally, diseases among cultured fish will result in poor growth and food conversion, which in turn increases production costs and interrupts production schedules (Hedrick, 1998). It is well known that fish diseases are not necessarily isolated events but are the end result of the relationship between pathogen, host and environment. A balanced relationship leads to good health and growth and a poor one to disease (Sanmartin Durán *et al.*, 1991). The severity of the disease is dependent upon the interaction of numerous variables including, but not limited to, the host, the pathogen and the environment, labelled the "triad". This triad is described as three interlocking sections with disease occurring at the intersection (Martin *et al.*, 1987; Thrusfield, 1995; Hedrick, 1998).

Parasites often cause little damage to fish in their natural habitat; however, their presence in the aquaculture environment may result in disease, pathological changes, decreased condition and/or reduction in market value of the cultured species (Dickerson and Clark, 1998; Scholz, 1999; Kent, 2000; Jones, 2001; Buchmann and Lindenstrom, 2002). Mortality or morbidity of parasitised fish may occur due to osmoregulatory disturbances (Grimnes and Jakobsen, 1996), pathological changes (Dezfuli *et al.*, 2002), immunosupression (Scharsack *et al.*, 2003), secondary infections (Mustafa *et al.*, 2000) or stress (Bowers *et al.*, 2000).

For AGD the pathogen is *N. perurans* with susceptible hosts being certain fish species and the environment being the seawater that the fish are in. The interactions within and among the pathogen, host and environment are complex with many

variables involved (Thrusfield, 1995). The impact of this disease is dependent upon the interactions of these variables for the host, pathogen and environment.

Host factors such as host species, fish size, population size and nutritional status are considered to be present constantly (Hedrick, 1998). Although AGD has been observed in numerous fish species, Atlantic salmon appear to be the most susceptible of cultured species (Munday *et al.*, 2001). Furthermore, the increased stocking densities that are seen in aquaculture are also reported to contribute to an increased virulence of microorganisms (Murray and Peeler, 2005)

The pathogen *N. perurans* has only recently been identified as the causative agent of AGD (Young *et al.*, 2007) and as yet not a lot is known regarding this pathogen. Factors associated with the pathogen are known to include the delivery to the host, duration of exposure, infectivity and number of pathogens which all in turn influence the severity of the disease (Hedrick, 1998; LaPatra, 1998). Douglas-Helders *et al.* (2003) reported that *N. pemaquidensis* remained infective for up to 14 days with no host contact. Furthermore, transmission of AGD has been successful through co-habitation of affected salmon with naïve salmon (Zilberg and Munday, 2000) as well as the exposure of fish to freshly harvested *Neoparamoeba* spp. from gills of fish known to have AGD (Zilberg *et al.*, 2001). However, AGD has not been achieved when exposing fish to cultured *Neoparamoeba* spp. (Kent *et al.*, 1988; Howard *et al.*, 1993). Since the discovery of *N. perurans* there is a need to investigate the culturing of the pathogen and the infectivity of this cultured pathogen.

Environment would probably be the least defined of the disease triad (Hedrick, 1998) and environmental factors can often significantly contribute to disease outbreaks (Nowak, 1999). The two main environmental factors considered important to AGD outbreaks are salinity and temperature (Clark and Nowak, 1999; Munday *et al.*, 2001). Other factors that are thought to influence AGD outbreaks include rainfall (Munday *et al.*, 1993; Clark and Nowak, 1999; Nowak, 2001), dissolved oxygen (Clark and Nowak, 1999) and biofouling on cages (Tan *et al.*, 2002), although these are not considered as important as salinity and temperature.

1.7 Research objectives and specific aims

The objective of this thesis was to investigate possible compounds for an oral treatment for AGD in Atlantic salmon.

1.7.1 Specific aims

Bithionol has previously been reported as amoebicidal at 1 and 10 mg L^{-1} over a period of six days and this study examined the toxicity of bithionol and bithionol sulphoxide to *Neoparamoeba* spp. at varying concentrations. It was hypothesised that bithionol would be amoebicidal at a wider range of concentrations than the 1 and 10 mg L^{-1} previously examined and that bithionol sulphoxide would exhibit similar amoebicidal tendency as that seen with bithionol. This was achieved through using the previously developed *in vitro* toxicity assay with 72 h duration and comparing bithionol and bithionol sulphoxide with seawater (positive control), alumina (particulate control) and the current freshwater mitigation (negative

control). The results were expected to provide compounds that were toxic *in vitro* to *Neoparamoeba* spp. that could then been examined *in vivo* for efficacy as an AGD treatment and serve as a basis for the research of subsequent chapters.

It was hypothesised that bithionol would be efficacious as a bath treatment for AGD whilst being non-toxic to Atlantic salmon. The efficacy and toxicity of bithionol to both fresh and seawater AGD-affected and non-affected Atlantic salmon and rainbow trout; when administered as a maximum 3h bath treatment at varying concentrations *in vivo* was assessed in laboratory trials. This was to identify the possibility of using bithionol to either improve or replace the current commercial freshwater mitigation strategy. Pathology, blood plasma electrolytes, tissue SDH and Na^+/K^+ ATPase as well as time to morbidity were quantified to assess the toxicity of bithionol on Atlantic salmon and rainbow trout in fresh and seawater.

Bithionol was assessed for its potential in alleviating AGD in Atlantic salmon. Bithionol was examined *in vivo* as an in-feed treatment to evaluate and describe the efficacy and toxicity of bithionol when administered orally at 25 mg kg^{-1} feed to Atlantic salmon 14 days prior to and 28 days post *Neoparamoeba* spp. exposure. Fish pathology, blood osmolality, and AGD parameters along with the feed intake of medicated feed pellets were quantified to assess the effects of bithionol as an oral treatment for AGD in Atlantic salmon. It was hypothesised that bithionol would be biotransfomed enabling its excretion across the gills which would in turn reduce the severity of AGD without any significant physiological consequences. The results

were expected to demonstrate the potential of bithionol as either an alternative or combined mitigation strategy for AGD.

Bithionol was assessed for its potential in alleviating AGD in Atlantic salmon in conjunction with the current freshwater bath mitigation. Prophylactic and therapeutic oral administration of bithionol at 25 mg kg^{-1} feed was assessed prior to and post freshwater bath. Fish pathology, including gross gill score, percent lesioned filaments, lesion size, specific growth rate, feed intake, condition factor and feed conversion ratio, were quantified to assess the effects of treatment administration. It was hypothesised that prophylactic administration of bithionol would provide an enhanced treatment efficacy and that, when combined with a freshwater bath, bithionol would provide a cumulative effect of treatment. The results were expected to highlight the most effective strategy for the oral administration of bithionol and elucidate the effectiveness of combining oral bithionol therapy and freshwater bath administration.

This chapter provides a summation of the main results and conclusions from all research chapters. The results and conclusions are discussed in terms of their relevance in light of the current literature regarding bithionol and its current uses as well as the development and use of treatments for AGD and other diseases such as sea lice. Consideration is given to future directions in regard to bithionol pharmacokinetics, environmental impacts, as well as combination therapy with freshwater and field trials.

CHAPTER 2

BITHIONOL AND BITHIONOL SULPHOXIDE EFFICACY *IN VITRO*

Submitted to Diseases of Aquatic Organisms

2 *In vitro* efficacy of bithionol and bithionol sulphoxide to *Neoparamoeba* spp. the causative agent of amoebic gill disease (AGD)

Renée L. Florent, Joy A. Becker, Mark D. Powell.

2.1 Abstract

The objective of the study was to evaluate the *in vitro* toxicity of bithionol and bithionol sulphoxide to *Neoparamoeba* spp. *Neoparamoeba* spp. are the causative agent of amoebic gill disease (AGD) and the current treatment for AGD-affected Atlantic salmon, *Salmo salar*, involving bathing sea-caged fish in freshwater for a minimum of 3 h. This process is labour intensive and the number of baths needed appears to be increasing; hence there is an effort to identify alternative treatments. Toxicity to *Neoparamoeba* spp. was examined *in vitro* using amoeba isolated from the gills of Atlantic salmon and exposing them to freshwater, alumina (10 mg L^{-1}), seawater, bithionol and bithionol sulphoxide at 0.1, 0.5, 1, 5 and 10 mg L^{-1}. The numbers of viable amoeba were counted using the trypan blue exclusion method at 0, 24, 48 and 72 h. Both bithionol and bithionol sulphoxide demonstrated *in vitro* toxicity to *Neoparamoeba* spp. at all concentrations examined. A similar toxicity to freshwater was observed with both chemicals at concentrations >5 mg L^{-1} following a 72 h treatment. Freshwater was the most effective with only 6% viable amoebae seen after 24 h and no viable amoeba observed after 48 h. Bithionol and bithionol sulphoxide were toxic to *Neoparamoeba* spp. at concentrations ranging from 0.1 to 10 mg L^{-1} over 72 h; however, freshwater still remained the most toxic with complete mortality seen at 48 h.

2.2 Introduction

Bithionol, 2,2'-thiobis (4,6-dichlorophenol), and bithionol sulphoxide, bis (2-hydroxy-3,5-dichlorophenyl) sulphoxide, are halogenated anthelminthics that are known to uncouple electron transport (Rew, 1978). They act on the mitochondrial respiratory chain (Iglesias *et al.*, 2002) and aid in the suppression of adenosine-5'-triphosphate (ATP) synthesis by uncoupling oxidative phosphorylation (Harder, 2002). They are effective against trematode and cestode infections in humans (Harder, 2002). Bithionol has been reported as effective for the treatment of metagonimiasis and paragonimiasis in humans and for killing the worms *in vitro* (Yokogawa *et al.*, 1961a; Yokogawa *et al.*, 1961b; Sawatari and Hamajima, 1967). Furthermore, bithionol is reported to kill the human parasite, *Entamoeba histolytica*, *in vitro* by inhibiting the endogenous and 2-propanol-supported respiration, but not the formation of ethanol in the parasite (Takeuchi *et al.*, 1984). Bithionol and bithionol sulphoxide have been examined as possible treatments for natural rumen fluke infection in cattle and tapeworm infections in cats, dogs, sheep and chickens (Enzie and Colglazier, 1960; Prasittirat *et al.*, 1997).

Both bithionol and bithionol sulphoxide have been examined as treatments for numerous fish parasites and showed mixed results. Santamarina *et al.* (1991) observed limited toxicity and complete *in vitro* efficacy against *Gyrodactylus* sp. in rainbow trout at 12.5 mg L^{-1}, with a minimum 20 mg L^{-1} reported as efficacious *in vivo*. Tojo *et al.* (1994b) stated that bithionol was efficacious *in vivo* against *Ichthyobodo necator* in rainbow trout at 25 mg L^{-1} for a 3 h freshwater bath on two consecutive days; however, higher concentrations exhibited some mortality. Finally,

Madsen *et al.* (2000) determined that bithionol at 0.1 mg L^{-1} was an effective treatment against trichodiniasis in European eels, *Anguilla anguilla*, but found bithionol to have a relatively narrow therapeutic index. More recently, bithionol has displayed efficacy for the treatment of *Neoparamoeba* spp., the causative agent of amoebic gill disease (AGD) (Florent *et al.*, 2007a; b).

In Tasmania, amoebic gill disease is the primary disease affecting the production of Atlantic salmon. The presumptive causative agent of AGD is a marine amphizoic protozoan parasite. Page (1987) had previously classified the aetiological agent as *Neoparamoeba pemaquidensis*. More recently another amoeba species *Neoparamoeba branchiphila* was cultured from AGD affected fish, but it was not known if one or both of these species were the aetiological agents of AGD (Dyková *et al.*, 2005). Furthermore, a new amoeba species designated *Neoparamoeba perurans* is believed to be the predominate aetiological agent of AGD affecting Atlantic salmon culture in Tasmania, invalidating previous classifications (Young *et al.*, 2007). *Neoparamoeba perurans* has recently been reported in Norway, highlighting the cosmopolitan nature of this pathogen (Steinum *et al.*, 2008)

Commercial mitigation of AGD uses a freshwater bath for 3 h, which is said to remove the amoeba and promote improved gill health (Parsons *et al.*, 2001a). However, the frequency of freshwater bathing has increased as it appears that each bath is proving to be less effective (Parsons *et al.*, 2001a). The process of freshwater bathing relies upon freshwater killing the amoebae and promoting their removal from the gills of AGD-affected fish. It has been reported that different water sources

have different treatment efficacies and these appear to correlate with water hardness or total ionic concentration (Clark, 2002). Of all chemicals screened as alternative treatments for AGD, only a small handful have achieve similar results seen with freshwater, including chlormaine-T (Harris *et al.*, 2004), hydrogen peroxide (Powell and Clark, 2003), levamisole (Findlay *et al.*, 2000) and bithionol (Powell *et al.*, 2003; Florent *et al.*, 2007b; 2007a).

The ability to study amoeba in isolation is useful in evaluating and identifying parameters affecting amoeba *in vivo*. Studies have been undertaken *in vitro* to determine the capacity of freshwater to inactive species of amoeba under different conditions (Howard and Carson, 1995; Powell and Clark, 2003). The aim of this study was to determine the effect of bithionol and bithionol sulphoxide on the survival of isolated gill amoebae *in vitro*. We hypothesised that both bithionol and bithionol sulphoxide would decrease the survival of isolated gill amoeba when compared to seawater.

2.3 Materials and Methods

2.3.1 Amoeba isolation by adherence

The isolation of *Neoparamoeba* spp. for toxicity assays followed the method develop by Morrison *et al.* (2004). Briefly, donor Atlantic salmon (AS) were obtained from an experimental AGD infection tank post-mortem (School of Aquaculture, University of Tasmania). All Atlantic salmon used displayed gross signs of AGD (raised white mucoid patches on the gills). Gill baskets were excised

from the AS, centrifuged at 400 *g* for 2 min in distilled water and rinsed with clean seawater three times, dislodging amoeba from the gills. The amoebae in seawater were allowed to adhere to Petri dishes for approximately 2 h at 18°C. Following, plates were washed with seawater and approximately 20 mL of seawater was added. The amoebae were allowed to adhere to Petri dishes overnight at 18°C. The adherent cells were removed by the addition of 1 mL Hanks balanced salt solution with trypsin and ethylenediaminetetraacetic acid (EDTA) (Appendix 1), washed, centrifuged at 400 *g* for 5 min and concentrated. An aliquot of amoeba isolate was stained with 0.5% trypan-blue-seawater mix at a dilution of 1:1 and live amoeba (those not taking up the stain) counts were determined using a haemocytometer (Neubauer, BS 748). Three replicate counts were made with 18 large squares counted per replicate.

2.3.2 In vitro toxicity assay

The *in vitro* toxicity assays used a modified version of the assay developed by Powell *et al.* (2003). The *in vitro* toxicity assay involved the use of previously isolated live amoeba which were adhered to flat bottom, 96 well plates and then exposed to different treatments. The amoeba solution was prepared by either concentrating or diluting to create approximately 10 000 cells in 150 µL per well and allowed to adhere for 1.5 h at 18°C.

Eight treatments were examined and assays ran for a period of 72 h. The number of live amoeba was determined at 0, 24, 48 and 72 h of exposure using the trypan-blue exclusion assay (described above). Times were chosen based on previous

literature (Powell *et al.*, 2003). All 96 wells were used allowing each treatment three replicates per day and each experiment was repeated eight times to give n equal to 24 per treatment. Survival of amoeba were calculated as a percentage of seawater control (conducted at the same time) to ensure consistency among treatments. For consistency with literature, the effective concentration (EC) at each time point was calculated. At each time point, concentration was plotted against percent survival for all, axes were logged (base 10 log) and a regression line was fitted. From this regression line, the time for 50% of the population to reach morbidity (EC_{50}) was determined for all treatments (Sprague, 1969).

2.3.3 Treatments

All test solutions were aerated to 100% air saturation and brought to 18°C before commencement of each experiment. Amoebae were exposed to either bithionol (Experiment 1) or bithionol sulphoxide (Experiment 2) (Sigma-Aldrich, Sydney, Australia) at 0, 0.1, 0.5 1, 5, or 10 mg L^{-1}. Concentrations were chosen to include and expand upon previous literature (Powell *et al.*, 2003). Bithionol and bithionol sulphoxide treatments were prepared making a stock solution using a mortar and pestle in order to create a suspension as they are both insoluble in water and then diluting to make the necessary concentrations. There were several controls examined including seawater (35‰, negative control), freshwater (de-chlorinated municipal source, positive control) and alumina at 10 mg L^{-1} (Sigma-Aldrich, particulate control).

2.3.4 Statistical Analyses

Statistical analysis was conducted using SPSS for Windows® (version 15.0). A one-way analysis of variance (ANOVA) was used to determine differences between assays. If no significant differences were found, assays were pooled and a two-way ANOVA was used to analyse means with time and treatment as factors. The interaction between time and treatment was examined first; if $p > 0.05$, there was an interaction and the factors of time and treatment were combined and a Tukey's post-hoc test conducted on the combined variable to identify where the differences occurred. Homogeneity was determined using a residual plot and Levene's test; where the data were not normally distributed or variance homogeneous, a square root transformation was used. A result was considered significant if $p \leq 0.05$ and results are presented as mean ± standard error of the mean (SEM). Survival of amoeba was calculated as a percentage of seawater control (conducted at the same time) to ensure consistency among treatments using the following equation.

$$\% \text{ of seawater control} = \left[\frac{\text{number of amoeba in treated group}}{\text{number of amoeba in seawater control group}} \right] \times 100$$

2.4 Results

Survival in seawater controls was equal to or better than initial concentrations observed at time 0 and are given as 100% survival. Amoebae survived when exposed to 10 mg L^{-1} alumina for the 72 h duration and were determined to be not significantly different from the seawater controls for both Experiment 1 and 2 ($F_{31,736} = 413.356$, $p < 0.001$, Fig 2.4-1, $F_{31,736} = 280.358$, $p < 0.001$, Fig 2.4-2,

respectively). Amoebae numbers declined rapidly when exposed to freshwater with a 94% relative reduction from the seawater control seen within the first 24 h in Experiment 1 and no viable amoebae were observed at any time points after time 0 (Fig 2.4-1). A similar result was seen in Experiment 2 with a 96% relative reduction in freshwater amoeba numbers from 0 to 24 h when compared to the seawater control (Fig 2.4-2). For both Experiment 1 and 2 EC_{50} values were calculated at each time point (Table 2.4-1).

Table 2.4-1. The effective concentration (mg L^{-1}) needed to cause 50% mortality (EC_{50}) in *Neoparamoeba* spp. when exposed to bithionol (BT) or bithionol sulphoxide (BTS) for 0, 24, 48 or 72 h.

	0 h	**24 h**	**48 h**	**72 h**
BT	n/a	7 mg L^{-1}	0.65 mg L^{-1}	0.32 mg L^{-1}
BTS	n/a	n/a	0.35 mg L^{-1}	0.11 mg L^{-1}

Note: Not applicable (n/a) indicates that 50% mortality had not occurred at the time point.

In Experiment 1 bithionol was effective at reducing amoebae numbers significantly at all concentrations and time points ($F_{31,736} = 413.356$, $p < 0.001$, Fig 2.4-1). When amoebae were treated with 10 mg L^{-1} of bithionol, there was a 53% relative reduction from 0 to 24 h when compared to seawater and by the culmination of the assay there was a final reduction of amoeba similar to that seen with freshwater. For bithionol at 5 mg L^{-1} a 95% relative reduction from seawater in amoeba numbers was observed which is similar to freshwater and bithionol at 10

and 1 mg L^{-1} following a 72 h treatment (Fig 2.4-1). There was a continuing decrease in amoeba numbers as time progressed across all bithionol treatments. At the culmination of the assay, the greatest reduction in amoeba numbers from seawater of 100, 95 and 92% was seen in the 10, 5 and 1 mg L^{-1} treatments, respectively. Both the 0.5 and 0.1 mg L^{-1} significantly reduced amoeba number compared to the seawater control; however the relative percent reduction at 72 h was 87 and 82%, respectively, which was significantly higher than the other bithionol treatments ($F_{31,736} = 413.356$, $p < 0.001$, Fig 2.4-1).

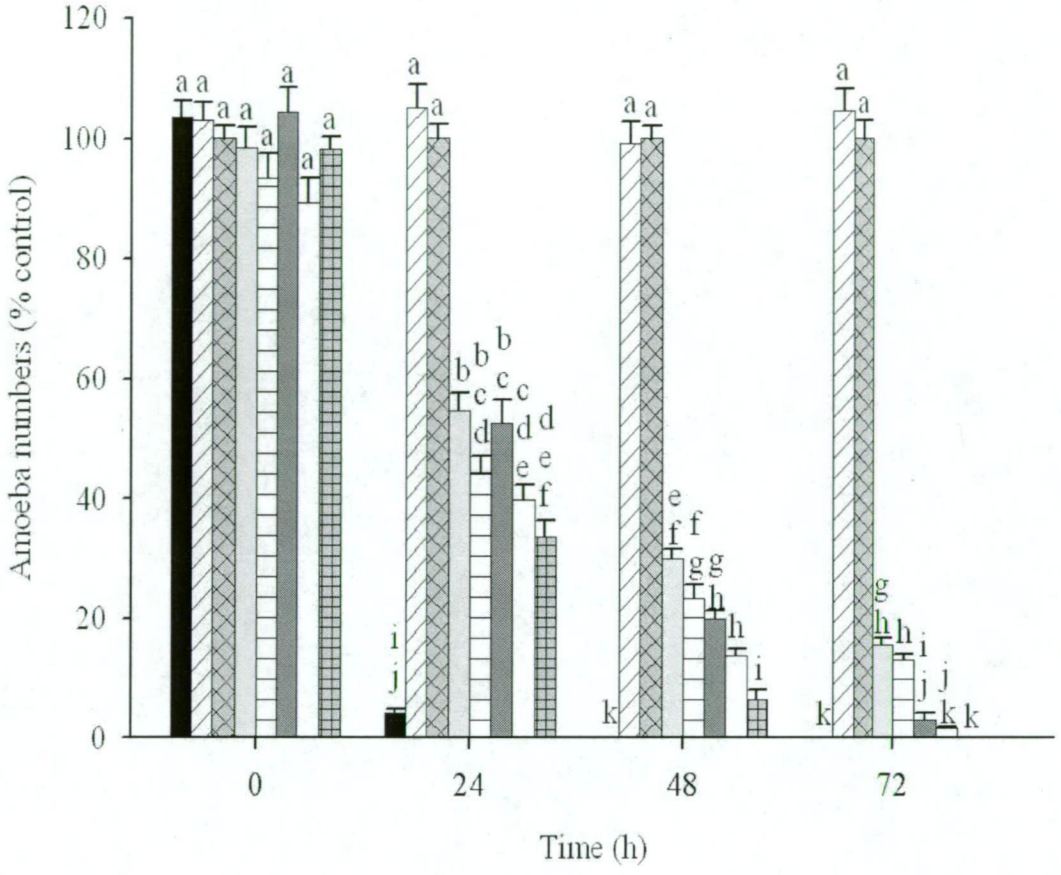

Fig 2.4-1. Effect of time and treatment on mean (± SEM) number of viable isolated amoeba (as a percentage of the seawater control) when exposed to either freshwater (■), 10 mg L^{-1} alumina (▨), seawater (▩), or bithionol at 0.1, 0.5, 1, 5 and 10 mg L^{-1} (▭, ▭, ▭, ▭, and ▦ respectively) (n = 24). Seawater controls remained at 10 000 cells $well^{-1}$ and are presented as 100% survival. Common letter across both time and treatment indicates no significant different using a Tukey's test ($p > 0.05$).

Bithionol sulphoxide examined in Experiment 2 reduced amoebae numbers significantly when compared to the seawater control at all concentrations and time points ($F_{31,736} = 280.358$, $p < 0.001$, Fig 2.4-2). When treated with 10 mg L^{-1} there was a 57% relative reduction compared to seawater in amoeba from 0 to 24 h and by the culmination of the assay this relative reduction of amoebae was 99% when compared to seawater, which was similar to that seen with freshwater. For the 5 mg L^{-1} treatment there was a 96% relative reduction in amoeba compared with seawater, which is similar to freshwater and bithionol at 10 and 1 mg L^{-1} following a 72 h treatment (Fig 2.4-2). Similar to bithionol, there was a continuing decrease in amoeba numbers as time progressed across bithionol sulphoxide treatments with the exception of the 0.5 and 0.1 mg L^{-1} treatments. At the culmination of the assay, the greatest reduction in amoeba when compared to the seawater control of 99, 96 and 91% was seen in the 10, 5 and 1 mg L^{-1} treatments, respectively. Both the 0.5 and 0.1 mg L^{-1} significantly reduced amoeba numbers compared to the control. However, the relative percent reduction at 72 h was 59 and 60%, respectively, which was significantly higher than the other bithionol treatments ($F_{31,736} = 280.358$, $p < 0.001$, Fig 2.4-2). Both the 0.5 and 0.1 mg L^{-1} treatments appeared to plateau out after 48 h with no further decrease observed after 72 h.

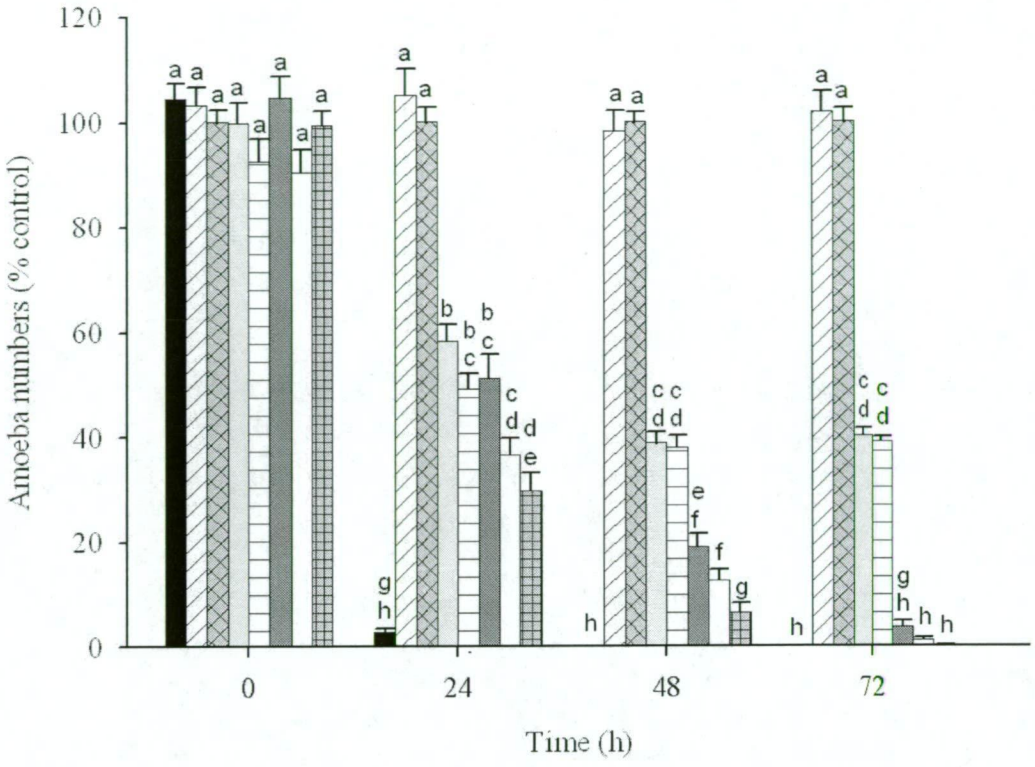

Fig 2.4-2. Effect of time and treatment on mean (± SEM) number of viable isolated amoeba (as a percentage of the seawater control) when exposed to either freshwater (■), 10 mg L^{-1} alumina (▨), seawater (▩), or bithionol sulphoxide at 0.1, 0.5, 1, 5 and 10 mg L^{-1} (□, ▭, ■, □, and ▦ respectively) (n = 24). Seawater controls remained at 10 000 cells $well^{-1}$ and are presented as 100% survival. Common letter across both time and treatment indicates no significant different using a Tukey's test ($p > 0.05$).

2.5 Discussion

Across all *in vitro* toxicity assays seawater controls were equal to or better than initial controls indicating that conditions were appropriate to observe growth. Present investigations identified that bithionol and bithionol sulphoxide used *in vitro* were successful at reducing amoeba numbers relative to seawater controls. When examining the alumina (particulate control) amoeba numbers did not differ from the seawater controls, indicating that the particulate matter in the treatment did not adversely affect amoebae survival over a 72 h period which is consistent with other studies (Powell *et al.*, 2003). The lowest dose able to kill a significant number of amoeba (>50%) was 0.1 mg L^{-1} and 1 mg L^{-1} for bithionol and bithionol sulphoxide, respectively.

In Experiment 1 and 2 when comparing freshwater to the seawater controls a 94 and 96% relative reduction were observed, respectively. The amoeba numbers declined rapidly in freshwater which is similar to other studies (Howard and Carson, 1993; Powell and Clark, 2003) indicating that freshwater was the most effective fast acting treatment. However, following the 72 hr toxicity assay bithionol successfully reduced amoebae numbers to similar levels as freshwater at concentrations of 5 and 10 mg L^{-1}. A similar result was seen with bithionol sulphoxide at 1, 5 and 10 mg L^{-1}, where amoebae numbers were reduced to a similar level as that seen with freshwater. Bithionol when used at 0.1, 0.5 or 1 mg L^{-1} was successful in reducing amoebae numbers albeit not as effective as freshwater or higher bithionol concentrations. Bithionol sulphoxide at the lower concentrations of 0.1 and 0.5 mg L^{-1} were the least effective reducing amoebae

numbers by 50%. However, even reducing the amoebae numbers by half would theoretically half the number of baths. When examining the cost of bithionol and bithionol sulphoxide the price difference is considerable with bithionol eight times more expensive than bithionol sulphoxide. Thus bithionol sulphoxide if as effective as bithionol is a significantly cheaper alternative.

To date, the only commercially viable and effective treatment for AGD is freshwater bathing. Several compounds have been screened for *in vitro* toxicity including various antimicrobials, antiparasitic, disinfectants and detergents with varying success. Of all chemicals screened only a small handful have achieved similar results seen with freshwater, including chlormaine-T (Harris *et al.*, 2004), hydrogen peroxide (Powell and Clark, 2003), levamisole (Findlay *et al.*, 2000) and bithionol (Powell *et al.*, 2003; Florent *et al.*, 2007b; 2007a). However, for some of these treatments they were most effective when added to a freshwater bath, hence still maintaining reliance upon freshwater bathing to treat AGD.

Studies examining the chemical control and treatment of *Neoparamoeba* spp. (Powell *et al.*, 2003), *Hexamita salmonis* (Tojo and Santamarina, 1998a), *Gyrodactylus* spp. (Tojo and Santamarina, 1998c), *Ichthyobodo necator* (Tojo *et al.*, 1994a; Tojo and Santamarina, 1998b), *Microcotyle sebastis* (Kim and Choi, 1998), *Pseudodactylogyrus* spp. (Buchmann *et al.*, 1992) and *Trichodina jadranica* (Madsen *et al.*, 2000) have all examined bithionol and in some cases bithionol sulphoxide *in vitro* and *in vivo* as bath and oral treatments. When examining the literature, studies show that bithionol exhibited mixed results of toxicity; however,

it was reported to be effective *in vitro* and *in vivo* as both bath and oral treatments, successfully reducing the parasite load. However, with the scale and intensity of salmonid farming occurring in Australia, a bithionol bath treatment would be impractical, as it is insoluble and would require a large amount of the compound to treat the bath water, thus making it labour and cost intensive. On the other hand, with the determination of toxicity to the target animal and its efficacy with respect to AGD it would be possible to incorporate bithionol in-feed and assess as a treatment for the control of AGD in Atlantic salmon. Furthermore, with the increasing need for the use of chemotherapeutants in aquaculture, it is very important to minimise the accumulation of chemicals in food for human consumption and the effect on the environment. Producing an in-feed treatment would assist in alleviating the release of large amounts of the compound into the surrounding water (Findlay *et al.*, 2000).

Bithionol and bithionol sulphoxide have been used as a successful treatment for numerous human disease including paragonimiasis (oriental lung fluke) and fascioliasis (liver fluke) (Yang and Lin, 1967; Bacq *et al.*, 1991). However, bithionol sulphoxide was found to have stronger anthelminthic activity than bithionol when examined in rats (Meshi *et al.*, 1970). Interestingly in this study, bithionol and bithionol sulphoxide exhibited similar reductions at high concentrations; however, at concentrations 0.5 mg L^{-1} or lower bithionol sulphoxide was not as effective as bithionol. Bithionol and bithionol sulphoxide have also been examined as possible treatments for natural rumen fluke infection in cattle and tapeworm infections in cats, dogs, sheep and chickens (Enzie and Colglazier, 1960;

Prasittirat *et al.*, 1997). *Neoparamoeba* spp. were susceptible to both bithionol and bithionol sulphoxide with median effective concentration (EC_{50}) at 48 h of 0.35 and 0.65 mg L^{-1} respectively and 0.11 and 0.32 mg L^{-1} at 72 h. The cladoceran, *Daphnia magna*, and the rotifer, *Brachionus calyciflorus*, were both found to be susceptible to bithionol with 48 h and 24 h EC_{50} values of 0.063 and 0.3 mg L^{-1} respectively (Yoshimura and Endoh, 2005). Bithionol was identified as toxic to medaka, *Oryzias latipes*, with a 96 h median lethal concentration (LC_{50}) of 0.24 mg L^{-1}, toxicity will be analysed further in Chapter 3 with the indication that medaka's LC_{50} is well within the therapeutic range in salmonids. In order for bithionol or bithionol sulphoxide to be administered as a treatment it would need to be licensed. Bithionol is used as an ingredient in deodorants, shampoos and surgical soaps, however has been banned from these products by the U.S. Food and Drug Administration (FDA) for safety reasons (Anonymous, 2002). However, it has been used as a parasiticide for cattle, chicken and sheep (Enzie and Colglazier, 1960; Mourot *et al.*, 1987). Bithionol is currently an investigatory drug for the Centre for Disease Control in America for therapeutic use in treating human paragonimiasis and fascioliasis (Anonymous, 2008). It is not currently licensed in Australia for use in animals or humans and in order to obtain a license a lot more research into the pharmacokinetics, tissue and blood residues and human impacts is required.

The screening of compounds *in vitro* for toxicity toward *Neoparamoeba* spp. enables the identification without considerable input of possible AGD treatment candidates. Stage I of drug screening, the development of single- and multi-day *in vitro* toxicity assays, has allowed for the bulk testing of numerous disinfectants,

antibiotics and antiprotozoal drugs. To date, few candidate drugs have moved to Stage II of testing, whereby salmon are offered drug-coated feed and subsequently experimentally challenged with *Neoparamoeba* spp. to determine fish toxicity and efficacy for preventing clinical signs of AGD. Even fewer have moved to Stage III testing involving field trials. This three-tiered approach has allowed for a strategic system for screening and identifying candidate drugs from a large pharmacopeia whilst maintaining effective resource management.

Bithionol has been identified as successful in stage I and II of the three-tiered approach mentioned above. However, it would be advantageous to examine the *in vitro* effects of bithionol and bithionol sulphoxide on *Neoparamoeba* spp. in more detail especially looking at shorter times and higher concentrations. In order to continue through to stage III involving field testing it would be advantageous to examine the pharmacology and residue levels of bithionol and its metabolites. Previous studies show that bithionol may work more effectively when high infections pressure is observed such as that in a laboratory trial as opposed to a low infection pressure seen typically in a farm situation (Chapter 5). Thus examining the efficacy of both bithionol and bithionol sulphoxide at lower infections pressure would be beneficial and aid in collating data to apply for an experimental license.

2.6 Acknowledgements

This research formed part of a project of the Aquafin CRC; and received funds from the Australian Government's CRC Programme, Fisheries R & D Corporation and other CRC participants.

CHAPTER 3

BITHIONOL BATH TOXICITY AND EFFICACY

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3 Evaluation of bithionol as a bath treatment for amoebic gill disease caused by *Neoparamoeba* spp.

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3.1 Abstract

This study examined the toxicity of bithionol to Atlantic salmon, *Salmo salar*, and rainbow trout, *Oncorhynchus mykiss,* in fresh and seawater and the efficacy of bithionol as a 1 h seawater bath treatment for amoebic gill disease (AGD). To examine toxicity, fish were bathed for 1, 3 and 6 h in bithionol, an anti-protozoal at 0, 1, 5, 10, 25 and 35 mg L^{-1} with toxicity determined by time to morbidity. Efficacy was examined by bathing AGD-affected Atlantic salmon and rainbow trout for 1 h at bithionol concentrations of 1 to 25 mg L^{-1}. Efficacy was determined by examining gill amoeba counts and identifying percent lesioned gill filaments at 1 and 24 h after bath exposure to bithionol. For both species, bithionol was determined to be toxic at 25 and 35 mg L^{-1} exhibiting median lethal times (LT_{50}) ranging from 21 to 84 min. Morbidity occurred in the 5 and 10 mg L^{-1} treatments. However, due to the sampling regime there were not enough fish available to calculate LT_{50}. Only bithionol at 1 mg L^{-1} was considered non-toxic with no signs of morbidity. Bithionol appeared to be more toxic in seawater than freshwater and had no acute effects on gill Na^+/K^+ ATPase and succinic dehydrogenase (SDH), or plasma osmolality and chloride concentration. Bithionol at 1 mg L^{-1} reduced percent lesioned gill filaments in Atlantic salmon and rainbow trout by 33 and 27% respectively, compared to the seawater control. Similarly, numbers of amoeba were

reduced by 33 and 43% for Atlantic salmon and rainbow trout respectively, when compared to the seawater control. Furthermore, bithionol reduced percent lesioned gill filaments as much as did the current industry standard of freshwater. This study demonstrated that a 1 h seawater bath containing 1 mg L^{-1} bithionol could be an improvement to the current method of treatment for AGD-affected Atlantic salmon and rainbow trout.

3.2 Introduction

Aquaculture involves intensive animal husbandry, maintaining large numbers of animals in a relatively limited space; these large numbers and limited space have lead to an increased risk of disease outbreaks (Stoffregen *et al.*, 1996). Parasites are provided with optimal conditions, as culture conditions contribute to rising water temperature and poor water quality, thus leading to significant fish loss (Schmahl *et al.*, 1989). Hence, the ongoing need for control of such diseases is paramount. Recent investigations have focused on antiprotozoals to keep farmed fish such as rainbow trout and Atlantic salmon disease free (Santamarina *et al.*, 1991). Successful elimination of endoparasites, such as nematodes and cestodes, has been achieved using oral administration of drugs including levamisole and praziquantel, whereas bath administration is generally used in treating ectoparasites, such as a formalin bath to treat trichodinads and monogeneans (Noga, 2000).

Significantly problematic to Australian salmon aquaculture, amoebic gill disease is caused by the protozoan parasite *Neoparamoeba* spp. (Munday *et al.*, 2001). *Neoparamoeba* spp. is a free-living marine amphizoic amoeba that attaches itself to

the gills particularly the secondary lamellae (Adams and Nowak, 2003). It is characterised macroscopically by the presence of raised, white mucoid patches with histological presentation of single or multi-focal epithelial hyperplasia leading to lamellar fusion (Parsons *et al.*, 2001a; Adams *et al.*, 2004). Freshwater bathing of affected fish is the current commercial management strategy for AGD; it lowers gross gill lesions, mortalities and is environmentally friendly (Munday and Zilberg, 2003); however, it is labour and cost intensive as well as stressful to the fish (Parsons *et al.*, 2001a; Munday and Zilberg, 2003). Attempts to identify potential chemotherapeutic agents have been limited due to either target fish toxicity or the cost of treatment (Alexander, 1991; Howard and Carson, 1994). Toxicity of several compounds to *Neoparamoeba* spp. has been examined *in vitro* including levamisole (Howard and Carson, 1995), chlorine dioxide, chloramine-T, hydrogen peroxide (Powell *et al.*, 2003; Powell and Clark, 2003), amprolium, albendazole, toltrazuril, and bithionol (Powell *et al.*, 2003). Howard and Carson (1994) reported that levamisole at concentrations $\geq$ 10 ppm *in vitro* were lethal to *N. pemaquidensis* and chloramine-T concentration of 25 and 50 ppm effectively reduced amoeba numbers to deionised water equivalents after 2 h (Powell and Clark, 2003). Powell *et al.* (2003) found amprolium at 1 mg L^{-1} and bithionol at 1 and 10 mg L^{-1} to be amoebicidal *in vitro*. From these studies, it was stated that chloramine-T and bithionol showed promise and would be suitable AGD treatments for *in vivo* examination (Powell and Clark, 2003).

Bithionol has been examined *in vitro* and *in vivo* as a bath treatment for other salmonid parasites, such as *Gyrodactylus* sp. and *Ichthyobodo necator*. Santamarina

et al. (1991) observed limited toxicity and complete *in vitro* efficacy against *Gyrodactylus* sp. in rainbow trout at 12.5 mg L^{-1}, with a minimum 20 mg L^{-1} reported as efficacious *in vivo*. Tojo *et al.* (1994b) stated that bithionol was efficacious *in vivo* against *I. necator* in rainbow trout at 25 mg L^{-1} for a 3 h bath on two consecutive days, higher concentrations exhibited some mortality. Finally, Madsen *et al.* (2000) determined that bithionol at 0.1 mg L^{-1} was an effective treatment against trichodiniasis in European eels, *Anguilla anguilla*, but found bithionol to have a relatively narrow therapeutic index.

This study aimed to determine the efficacy of bithionol as a bath treatment for AGD. The first objective was to evaluate the toxicity of bithionol administered via a bath treatment to Atlantic salmon and rainbow trout held in either fresh or seawater using concentrations between 1 and 35 mg L^{-1}. The second objective was to evaluate the efficacy of bithionol at 1 to 25 mg L^{-1} as a bath treatment for AGD-affected Atlantic salmon and rainbow trout.

3.3 Materials and Methods

3.3.1 Toxicity Study

3.3.1.1 Fish Husbandry

Juvenile diploid mixed sex rainbow trout (RBT), with a mass of 74.5 ± 1.0 g and a fork length of 18.5 ± 0.1 cm (N=234) were obtained from Sevrup Fisheries (Tasmania, Australia). Atlantic salmon (AS) diploid mixed sex spring smolts, with a mass of 74.1 ± 0.9 g and a fork length of 18.8 ± 0.1 cm (N = 234) were obtained

from SALTAS salmon hatchery (Tasmania, Australia). Both groups of fish were maintained at the University of Tasmania Aquaculture Centre for a minimum of three weeks prior to experimental use. Fish were housed in two 3000 L Rathburn tanks with recirculated water and individual biofilter systems. Half of each species were housed in freshwater (de-chlorinated municipal source, 15.5 ± 1.0°C) and the other half of each species were maintained in seawater (35‰, 1 µm filtered, 15.5 ± 1.0°C). The tanks received constant aeration and oxygen levels were monitored daily using a Handy Gamma Oxy Guard (Birkerød, Denmark). Fish were fed twice daily to satiation and feed was withheld one day prior to bath administration. Immediately prior to bath treatment, nine fish were sampled as pre-experimental controls (see below).

3.3.1.2 Bath Administration

Bithionol concentrations of 0, 1, 5, 10, 25, and 35 mg L^{-1} were examined for Atlantic salmon and rainbow trout toxicity using a maximum 6 h bath duration. Toxicity was examined in both freshwater (fw) (municipal source, 15.5°C, pH 7) and seawater (sw) (35‰, 15.5°C, pH 8.2). Baths were conducted in triplicate with six fish in 20 L plastic tubs at stocking densities ranging between 19.8 and 24.4 g L^{-1}. Tubs received constant aeration, with oxygen levels monitored every 15 min and maintained at 95% saturation using oxygen, if required. Temperature, salinity, pH, ammonia, nitrate and nitrite were measured hourly throughout bath duration. Bithionol (Sigma-Aldrich Pty. Ltd, Castle Hill, Australia) is insoluble in water, so it was administered as a suspension prepared using 1 mL of bath water and a mortar and pestle (Tojo *et al.*, 1994b), with some precipitation occurring at

concentrations > 10 mg L^{-1}. In all cases, fish were euthanized using clove oil at a concentration of 0.02% w/v. All procedures were conducted in accordance with the Australian code of practices for the care and use of animals for scientific papers (7^{th} edition) under administration of the University of Tasmania Animal Ethics Committee.

3.3.1.3 Data Collection

Fish were sampled (two fish per tub) at 1, 3 and 6 h, unless fish were identified as moribund. Morbidity was determined as fish that were swimming slowly, close to the water surface or exhibiting loss of equilibrium. For each fish, mass, fork length and if appropriate time to morbidity were recorded and blood was sampled for plasma analysis. Gill tissue was processed for histology and biochemical analysis (see below) and liver tissue was processed for histology. Immediately following collection, blood was centrifuged for 2 min at 8 000 x *g* using a Spinwin MC-01 (Tarsons Products Pty. Ltd. Minto, Australia), the serum was decanted and frozen. Gill tissue was snap frozen in liquid nitrogen for biochemical analysis and stored at -80°C until analysis could be performed. Plasma samples were thawed and osmolality determined using a Wescor Vapro 5520 vapour pressure osmometer (Helena Laboratories Pty. Ltd., Mount Waverly, Australia). Chloride levels were determined using plasma diluted 1000 X with deionised water following the spectrophotometric method of Zall *et al.* (1956).

Thawed tissue samples for the 0, 10 and 35 mg L^{-1} were examined for Na^+/K^+ ATPase and succinic dehydrogenase (SDH) activity. If differences were found

between the above treatments then other concentrations were examined. Na^+/K^+ ATPase activity was determined according to a method modified from McCormick (1993). Briefly, tissue was homogenized using an Eppendorf micro pestle homogeniser in ice-cold SEID (3X concentrate) (250 mM sucrose, 10 mM sodium ethylene-diaminetetraacetic acid (EDTA), 50 mM imidazole, 0.1 g sodium deoxycholic acid) and centrifuged at 5 000 x *g* for 30 seconds. ATPase activity was determined in the presence or absence of 0.5 mM ouabain (Sigma-Aldrich Pty. Ltd, Castle Hill, Australia) at 25°C and absorbance was measured at a wavelength of 340 nm for ten minutes at ten second intervals. Succinic dehydrogenase was determined using the spectrophotometric method as used by Powell *et al.* (2001).

For consistency with literature, the time for individuals to reach morbidity was determined lethal time (LT) assuming there was no recovery from morbidity. Time to morbidity was plotted against percent cumulative morbidity for all groups at 25 and 35 mg L^{-1}, axes were logged (base 10 log) and a regression line was fitted. From this regression line, the time for 50% of the population to reach morbidity (LT_{50}) was determined for all groups (Sprague, 1969). Median lethal times were obtained for bithionol concentrations 25 and 35 mg L^{-1} for fresh and seawater Atlantic salmon and rainbow trout as morbidity occurred prior to first sampling period.

3.3.1.4 Histology

The left gill basket was excised, rinsed gently in 0.2 µm filtered seawater and fixed in seawater Davidson's fixative and the liver was fixed in 10% neutrally

buffered formalin for 24 h and then transferred to 70% ethanol. The second left anterior hemibranch was removed, along with a small section of liver, dehydrated, embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin and eosin (H & E). The sections were viewed under a light microscope (Olympus, Hamburg, Germany) at X100 to X400 magnifications. Sections were examined for pathological changes including epithelial separation, aneurisms, change in pavement cells, chloride cells, hyperplasia, and inflammation (Mallatt, 1985; Takashima and Hibiya, 1994).

3.3.2 Efficacy Study

3.3.2.1 Fish Husbandry

Juvenile diploid mixed sex RBT, with a mass of 133.9 ± 6.0 g and a fork length of 24.0 ± 0.3 cm (N = 42) were obtained from Sevrup Fisheries. Atlantic salmon diploid mixed sex spring smolts, with a mass of 92.0 ± 2.4 g and a fork length of 20.3 ± 0.2 cm (N = 108) were obtained from SALTAS salmon hatchery. Following laboratory exposure to *Neoparamoeba* spp., (see below) all fish exhibited gross signs of AGD according to Munday *et al.* (2001). Atlantic salmon and RBT were housed separately in two seawater (35‰, 1 µm filtered, 17.0 ± 0.8°C) re-circulation systems, consisting of three 590 L tanks, a 500 L header and a 500 L sump providing a total system volume of 2770 L. The tanks received constant aeration and oxygen levels were monitored daily. Fish were fed twice daily to satiation and feed was withheld one day prior to bath administration. Immediately prior to bath treatment six fish were sampled as pre-experimental controls (see below).

3.3.2.2 Amoeba isolation and exposure

Naïve experimental AS and RBT were exposed to *Neoparamoeba* spp. according to Morrison *et al.* (2004). Briefly, eight donor Atlantic salmon were obtained from an experimental AGD infection tank post-mortem (School of Aquaculture, University of Tasmania, Launceston, Tasmania, Australia). Gill baskets were excised from the AS, centrifuged in distilled water, and rinsed with seawater three times, dislodging amoeba from the gills. The amoebae in seawater were allowed to adhere to Petri dishes for approximately 2 h at 18°C. Plates were then washed with seawater and approximately 20 mL of seawater was added. The amoebae were allowed to adhere to Petri dishes overnight at 18°C. The adherent cells were removed by the addition of 1000 µL Hanks balanced salt solution with trypsin and EDTA (Appendix 1), washed, centrifuged and concentrated. Live amoeba counts were determined using a haemocytometer (Neubauer, BS 748). Three replicate counts were made with 18 large squares counted per replicate. The isolation obtained 2 500 000 amoebae delivering a final concentration of approximately 300 cells L^{-1} per system.

Following bath treatments, *Neoparamoeba* spp. were re-isolated from the gills using a technique modified from Howard and Carson (1995) and Powell and Clark (2003). Briefly, the right gill basket was excised and rinsed gently in 0.2 µm filtered seawater and individual arches were scraped with a bacterial spreader to remove mucus. Mucus was collected in individual 50 mL centrifuge tubes and re-suspended in sterile seawater up to 10 mL. A 100 µL aliquot of mucus-amoeba suspension was sampled and stained with 0.05% trypan blue viability stain at a

dilution of 1:1. Live amoeba counts were determined using a haemocytometer. Three replicate counts were made with 18 large squares counted per replicate. The total number of live amoebae per fish was then calculated and divided by the natural log of the fish mass to account for scaling differences in gill surface area with fish of different mass (Palzenberger and Pohla, 1992).

3.3.2.3 Bath administration

To determine bath efficacy, AGD-affected Atlantic salmon and rainbow trout were exposed to either a 1 h seawater bath containing bithionol concentrations of 1, 5, 10, or 25 mg L^{-1}, a 3 h freshwater bath or a 1 h seawater bath containing alumina (25 mg L^{-1}, a particulate control). The alumina was prepared using the bithionol procedure described above. Baths were conducted in triplicate with six fish in 20 L plastic tubs at stocking densities ranging from 11.7 to 29.9 g L^{-1}. Following the bath, fish were returned to the 2770 L recirculation system (as described above) and monitored for morbidity until sampling occurred 24 h later. The baths were conducted using the procedure mentioned previously for toxicity trial.

3.3.2.4 Data Collection

Fish were sampled (two fish per tub) immediately following the bath and after 24 h, unless fish were identified as moribund. As in the previous study, for each fish mass, fork length and if appropriate time to morbidity were recorded and blood was sampled for plasma analysis. Gill tissue was processed for histology and amoeba counts. Immediately following collection, blood was centrifuged for 2 min at 8 000 x *g* using a Spinwin MC-01, the serum was decanted and frozen. Plasma

samples were thawed and osmolality determined using a Wescor Vapro 5520 vapour pressure osmometer.

3.3.2.5 Histology

The left gill basket was excised and processed for histology as described in section 3.3.1.4. The sections were viewed under a light microscope at X100 to X400 magnifications. The number of filaments exhibiting AGD lesions (Kent *et al.*, 1988) were counted and expressed as proportions of the total number of filaments in each section (Parsons *et al.*, 2001a) and presented as percent lesioned gill filaments. A filament was counted only when the central venous sinus was visible in at least two-thirds of the filament and lamellae were of equal length bilaterally present to near the tip of the filament (Speare *et al.*, 1997).

3.3.3 Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted using SPSS for Windows® (version 11.5). A two-way analysis of variance (ANOVA) was used to determine differences between fixed factors of sampling time and treatment. If $p \geq 0.05$, a two-way ANOVA was used to determine differences between fixed factors of day and treatment. If no significant differences were found, results were pooled and either a two-way ANOVA was used to determine differences between water and bithionol concentration or a one-way ANOVA used to determine differences between efficacy treatments. Homogeneity was determined using a residual plot and Levene's test; if the data were not normally distributed a log base 10 transformation was used. A result was

considered significant if $p \leq 0.05$. Relative percent reduction (RPR) was calculated for crude number of amoebae and percent lesioned gill filaments using the following equation.

$$RPR = 1 - \left[\frac{\text{number of amoeba in treated group}}{\text{number of amoeba in control group}} \right] \times 100$$

3.4 Results

3.4.1 Toxicity Study

Median morbidity times were determined in all groups at bithionol concentrations of 25 and 35 mg L^{-1} (Table 3.4-1). Atlantic salmon in freshwater at 25 and 35 mg L^{-1} had an LT_{50} of 86 and 44 min compared to seawater values of 35 and 21 min, respectively.

A similar trend was seen with RBT at 25 and 35 mg L^{-1} in freshwater having median lethal times of 51 and 46 min compared to seawater RBT of 29 and 22.5 min, respectively. For all groups, the number of moribund fish over the 6 h bath duration was recorded in Table 3.4-2. At concentrations of 25 and 35 mg L^{-1} all groups exhibited 100% morbidity within 3 h. One hundred percent morbidity was observed at 10 mg L^{-1} within 3 h in both fw and sw RBT, which was the same for sw AS but not fw AS, which had five (27%) moribund fish after 6 h. At 5 mg L^{-1} there were indications that bithionol may be more toxic in seawater with 12 (66%) and 5 (27%) RBT and AS moribund compared to freshwater with 1 (5%) and 0

RBT and AS moribund, respectively. There was no morbidity observed in the 0 and 1 mg L^{-1} treatments across all groups.

Table 3.4-1. Median lethal time and inter-quartile range (IQR) for seawater (sw) and freshwater (fw) Atlantic salmon (AS) and rainbow trout (RBT) exposed to a 1 hour bithionol bath at a concentration of 25 or 35 mg L^{-1}.

	Median lethal time (min ± IQR)			
Concentration (mg L^{-1})	**sw AS**	**sw RBT**	**fw AS**	**fw RBT**
25	35 ± 13	29 ± 10	86 ± 47	51 ± 15
35	21 ± 12	22.5 ± 11	44 ± 10	46 ± 15

Note: (n = 18)

With increasing bithionol concentration, plasma osmolality, plasma chloride, gill Na^+/K^+ ATPase activity and gill SDH were not significantly different for both species within each water source (Fig 3.4-1 and Fig 3.4-2) (all $p > 0.122$). This was comparable with histological results in which no pathology was seen in any of the treatments (histology not shown). As expected, plasma osmolality increased from freshwater to seawater observed in both species ranging from 30-100 mmol kg^{-1} ($p < 0.001$, Fig 3.4-1 ia, iia). Similarly, differences were found between freshwater and seawater in both AS and RBT gill Na^+/K^+ ATPase activity exhibiting an approximate 2.5 times increase across all treatments from fw to sw ($p < 0.001$, Fig 3.4-2. ia, iia).

Table 3.4-2. The number of moribund Atlantic salmon and rainbow trout in both freshwater (fw) and seawater (sw) observed at varying bithionol concentrations over a 6 hour (h) period.

Concentration (mg L^{-1})	Number of moribund (sw, fw)		
Atlantic salmon	**0-1 h**	**1-3 h**	**3-6 h**
0	0, 0	0, 0	0, 0
1	0, 0	0, 0	0, 0
5	4, 0	1, 0	0, 0
10	18, 0	na, 3	na, 2
25	18, 6	na, 12	na, na
35	18, 18	na, na	na, na
Rainbow trout			
0	0, 0	0, 0	0, 0
1	0, 0	0, 0	0, 0
5	2, 0	6, 1	4, 0
10	18, 4	na, 14	na, na
25	18, 9	na, 9	na, na
35	18, 18	na, na	na, na

Note: Not applicable (na) indicates there were no fish left at the time point (n = 18)

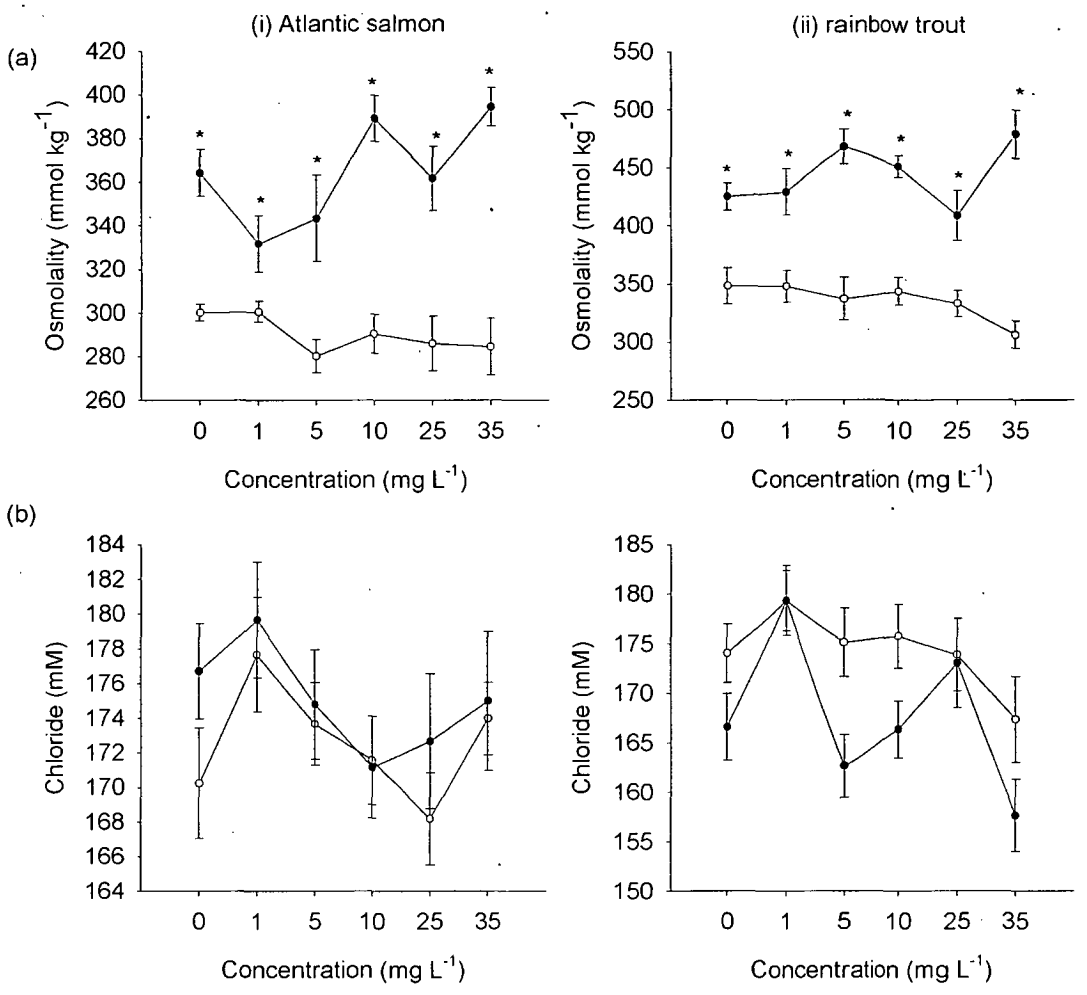

Fig 3.4-1. Mean (± SEM) (a) plasma osmolality and (b) plasma chloride for freshwater (open circle) and seawater (closed circle) (i) Atlantic salmon and (ii) rainbow trout when exposed to varying bithionol bath concentrations. Asterisks denotes a significant difference between fresh and seawater.

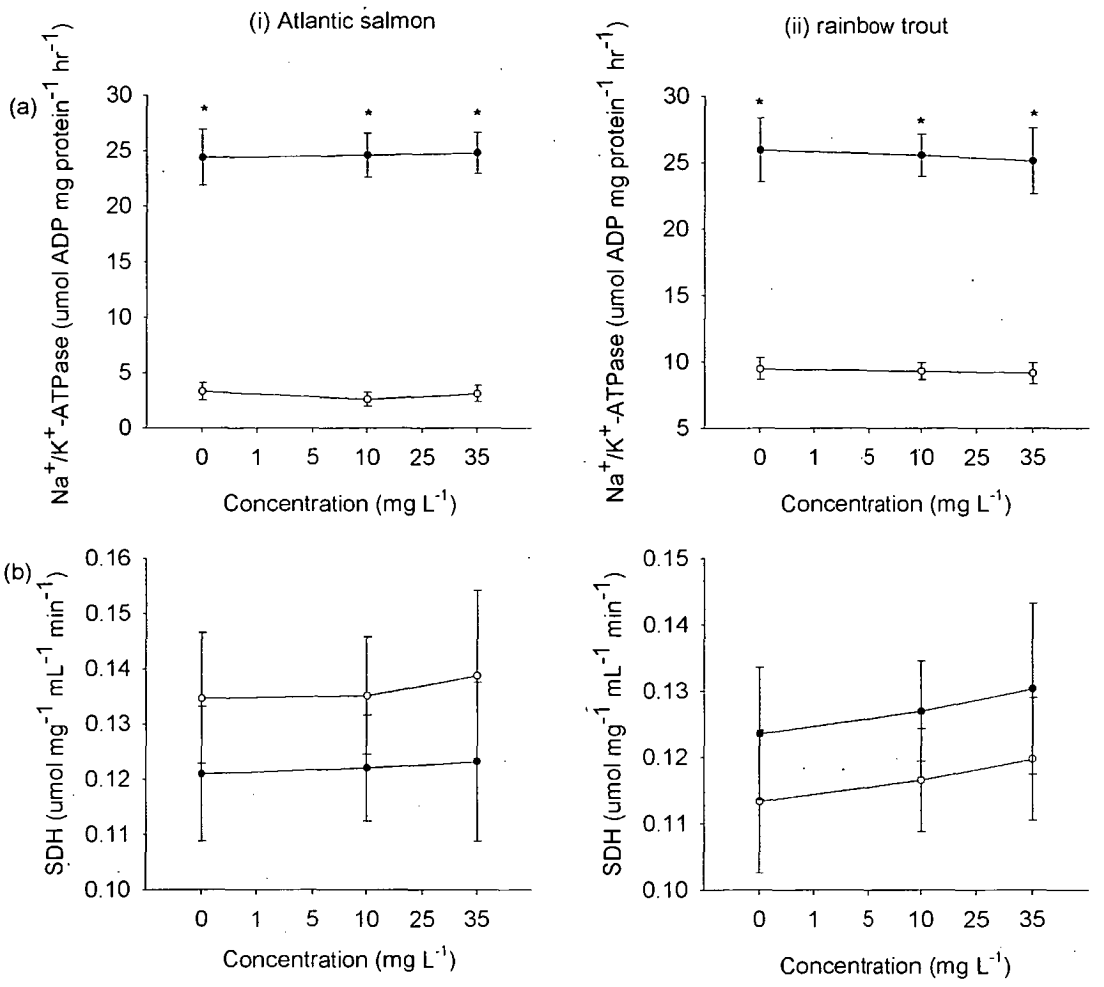

Fig 3.4-2. Mean (± SEM) (a) gill Na^+ / K^+-ATPase activity and (b) gill succinic dehydrogenase (SDH) activity for freshwater (open circle) and seawater (closed circle) (i) Atlantic salmon and (ii) rainbow trout when exposed to varying bithionol bath concentrations. Asterisks denotes a significant difference between fresh and seawater.

3.4.2 Efficacy

For both AS and RBT, the number of morbid fish over the 1 h bath duration and during the following 24 h was recorded in Table 3.4-3. During the 1 h bath AS and RBT exhibited 100% morbidity at 25 mg L^{-1}. Morbidity of 44% and 16% was observed during the first hour for both AS and RBT at 10 mg L^{-1}, respectively; however, no morbidity was reported in fw, 0, 1 and 5 mg L^{-1} treatments. Twenty four hours post bath, AS in the 10 mg L^{-1} treatment exhibited 44% morbidity, with no morbidity observed for other salmon. Whereas RBT had morbidity across all bithionol treatments, with one (16%) morbid fish in both the 1 and 5 mg L^{-1} treatments and four fish (66%) in 10 mg L^{-1}. There was no morbidity observed for RBT or AS in the 0 mg L^{-1} or freshwater treatments.

Table 3.4-3. The number of moribund Atlantic salmon (AS) and rainbow trout (RBT) with amoebic gill disease observed between 0 and 1 hour (0-1 h) and 1 and 24 hours (1-24 h) when exposed to either a freshwater control bath or a 1 hour bath at varying bithionol concentrations.

	Number of moribund Concentration mg L^{-1}					
0-1 h	**fw**	**0**	**1**	**5**	**10**	**25**
AS	0	0	0	0	4	9
RBT	0	0	0	0	1	6
1-24 h						
AS	0	0	0	0	4	na
RBT	0	0	1	1	4	na

Note: (AS n = 9, RBT n = 6) Not applicable (na) indicates no fish were left at time point.

There was a concentration dependant decrease in percent lesioned gill filaments in both Atlantic salmon and rainbow trout (Fig 3.4-3a). For AS, the lowest concentration of bithionol tested (1 mg L^{-1}) significantly reduced percent lesioned gill filaments to similar levels of the fw control, with all other bithionol concentrations having significantly reduced percent lesioned gill filaments in comparison to the sw control ($p < 0.001$, Fig 3.4-3a). A similar pattern was observed in RBT, with all bithionol concentrations significantly reducing percent lesioned gill filaments compared to the sw control, although they were not reduced to a similar level seen in the fw control (Fig 3.4-3a).

Concurrently, there was a bithionol concentration dependant decrease in amoeba numbers on the gills of AS and RBT following the 1 h bath ($p < 0.001$, Fig 3.4-3b). Atlantic salmon exposed to a 1 h bithionol bath at 1, 5, 10 or 25 mg L^{-1} had a relative percent reduction in amoeba numbers when compared to the sw control of of 33, 46, 47 and 60 %. respectively. Rainbow trout similarly at corresponding concentrations exhibited relative percent reductions of amoeba numbers compared to sw control of 43, 49, 56 and 60%, respectively. There were no differences in plasma osmolality at any of the treatment concentrations for both AS and RBT ($p > 0.078$, Fig 3.4-3c).

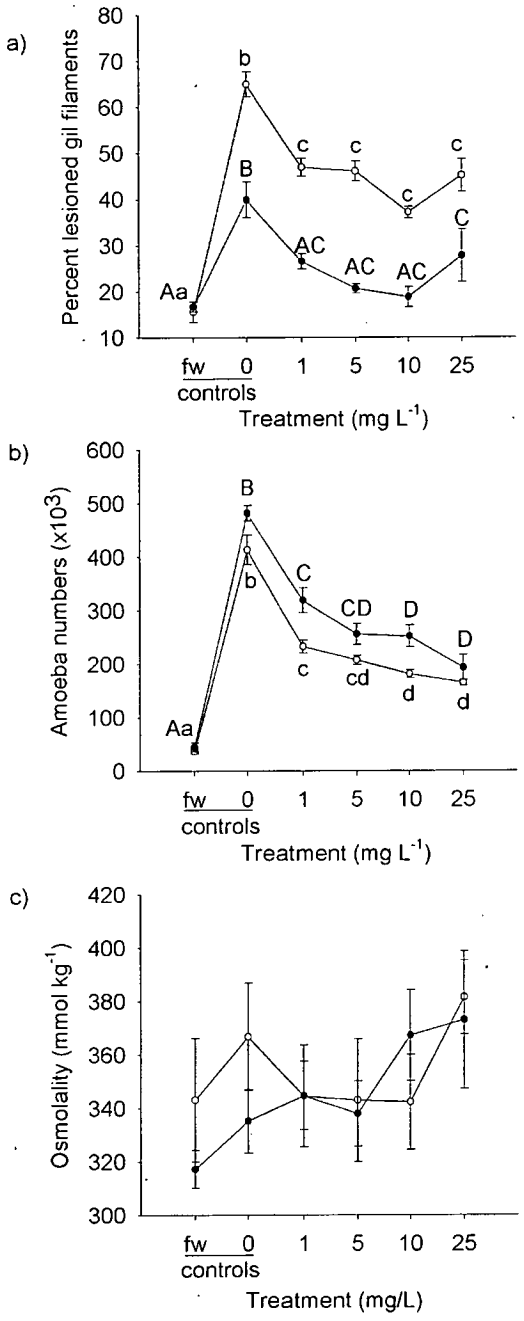

Fig 3.4-3. Mean (± SEM) (a) percent lesioned gill filaments, (b) crude amoeba numbers and (c) plasma osmolality for rainbow trout (open circle) and Atlantic salmon (closed circle) when administered varying bithionol bath concentrations. Lower and upper case letters denote significant differences within Atlantic salmon and rainbow trout, respectively.

3.5 Discussion

Bithionol concentrations greater than 25 mg L^{-1} were considered toxic with all fish exhibiting signs of morbidity within three hours. This was similar to results reported by Tojo *et al.* (1994b) and Santamarina *et al.* (1991) with significant levels of mortality reported in RBT at concentrations greater than 30 mg L^{-1}. In the toxicity trial, there were indications that bithionol in seawater may be more toxic than in freshwater, with LT_{50} values for both Atlantic salmon and rainbow trout equal to 44 to 84 min for freshwater compared with 21 to 35 min for seawater, respectively. Salinity has been reported to have little influence on the tolerance of fish to toxicants, but fish do tolerate toxicants better in conditions similar to which they reside in, possibly due to lessened osmotic problems (Sprague, 1990). This suggests that the increased toxicity with salinity may be linked to osmotic problems. Plasma osmolality was higher in seawater compared to freshwater for both species in the toxicity trial. However, no differences were seen in plasma chloride concentrations, which were above 160 mEq L^{-1} quoted as the minimum acceptable level for chloride ions by Morgan and Iwama (1991), indicating the fish were not likely to be suffering osmotic stress. Hence, further investigation would be required in order to determine the mechanism for the observed difference in bithionol toxicity between fish held in fresh and seawater. Bithionol concentration did not affect plasma osmolality or plasma chloride concentration, adding to the hypothesis that the cause of differential toxicity between freshwater and seawater was not related to osmoregulation.

The difference between fw and sw gill Na^+/K^+ ATPase levels obtained in this study correspond to those found by Pfeiler and Kirschner (1972) who showed Na^+/K^+ ATPase levels in rainbow trout to be 12.6 ± 1.7 μmol Pi mg protein^{-1} h^{-1} in freshwater and 23.6 ± 2.1 μmol Pi mg protein^{-1} h^{-1} in seawater. Similarly, Ventrella *et al.* (2001) reported that RBT had lower activity in fw compare to brackish water (22‰). The difference observed between fw and sw is attributed to the increase in Na^+ in marine fish, which must be pumped across the gills into the environment (Pfeiler and Kirschner, 1972). McCormick (1993) reported Na^+/K^+ ATPase to be an ion-translocating enzyme found in high concentrations in gill chloride cells and believed to be the primary enzyme for excretion of excess Na^+ and Cl^- from body fluids (Handeland *et al.*, 2004). Succinic dehydrogenase, a chloride cell activity marker, plays an important role in mitochondria as it participates in the electron transport, respiratory chain and Krebs cycle (Brière *et al.*, 2005). In both species, the highest concentration of bithionol did not inhibit gill Na^+/K^+ ATPase or gill SDH from the respective control group, indicating that bithionol concentration did not appear to impair gill ion regulatory function over the 6 h sample period. This was further evidenced by the absence of acute changes in plasma chloride concentration. No difference suggests that the two gill toxicity markers of SDH and Na^+/K^+ ATPase were not acutely affected by bithionol and the cause of toxicity was most likely not related to gill function.

Both Na^+/K^+ ATPase and SDH were examined because bithionol is known to be a metabolic disruptor, uncoupling electron transport in fish ciliates including *Tetrahymena pyriformis* (Griffin, 1989) and *Trichodina jadranica* (Madsen *et al.*,

2000). Takeuchi *et al.* (1984) postulated that bithionol functioned as an uncoupler of mitochondrial oxidative phosphorylation in the trophozoite stage of the *Entamoeba histolytica*, the main amoeba pathogenic to humans. It is suggested that bithionol has the ability to inhibit activity of fumarate reductase, malic enzyme, phosphogluconate dehydrogenase and succinate dehydrogenase in human liver and lung flukes (Hamajima *et al.*, 1979), although the inhibition of succinate dehydrogenase was not observed in this study. It is important to note that the assay used in this study examined Na^+/K^+ ATPase activity, not the presence of ATP, therefore showing that bithionol did not inhibit Na^+/K^+ ATPase in the gill. There was no indication of the availability of ATP to drive the Na^+/K^+ ATPase in chloride cells. Longer-term exposure may affect the availability of ATP, therefore in future studies it would be advantageous to examine the presence of ATP in gill tissue.

Histological sections of gill or liver tissue from all bithionol concentrations did not reveal any signs of alterations from the 0 mg L^{-1} treatment. Chloride cells, mucous cells, inflammation and epithelial separation were similar across all concentrations; reinforcing the suggestion that bithionol did not acutely affect the gill. Bithionol is known to be readily eliminated by the kidneys in humans and rats, but accumulates in bile of mice (Barrett-Connor, 1982). Therefore future studies should include sampling of the anterior and posterior kidney, in addition to the liver and gills.

Bithionol, at a minimum concentration of 1 mg L^{-1}, significantly reduced the percent lesioned gill filaments and numbers of gill amoebae in both Atlantic salmon

and rainbow trout compared to the seawater control fish. Moreover, the observed reduction in percent lesioned gill filaments in Atlantic salmon at bithionol concentrations of 1, 5 and 10 mg L^{-1} was equal to that of the current industry standard of a freshwater bath. Reduction in amoeba numbers after the commercial 3 h freshwater bath when compared to a sw control have been reported to be 85-90% (Clark *et al.*, 2003). A similar reduction of 90% from the sw control was seen in the efficacy trial with both AS and RBT freshwater baths. Atlantic salmon bathed in bithionol at concentrations from 1 to 25 mg L^{-1} exhibited a reduction in amoeba numbers from the sw control of 33% to 60%. Similarly, rainbow trout resulted in reductions of amoeba numbers from the sw control compared to 1 and 25 mg L^{-1} of 43% and 60% respectively. These results indicate that a 1 h bithionol bath at 1 mg L^{-1} in sw can be as efficacious as freshwater in treating AGD, as a minimum crude amoeba reduction of 33% was seen compared to no treatment.

Other treatments for AGD that have been considered *in vivo* include softened freshwater (contains fewer divalent cations, approx. 0.4 mM Ca^{2+}), which was suggested to alter mucus viscosity making it more efficacious than hard freshwater reducing amoeba numbers and alleviating the pathological signs of amoebic gill disease (Roberts and Powell, 2003a). Chloramine-T, a disinfectant at 10 mg L^{-1} in a seawater bath was as effective as a freshwater bath reducing amoeba numbers by approximately 60%, compared to the seawater control (Harris *et al.*, 2004). A similar reduction was obtained with a 1 h bithionol bath at 25 mg L^{-1}; however, it was associated with high morbidity. Additionally, levamisole, the levoisomer of tetramisole, is a recognised antihelmintic and has been reported as effective *in vitro*

at ≤ 50 mg L^{-1} for *Gyrodactylus* spp. (Schmahl *et al.*, 1989) and an effective treatment for AGD-affected Atlantic salmon at 50 mg L^{-1} (Munday and Zilberg, 2003). This indicates that levamisole is almost as efficacious as freshwater for treating AGD, but was associated with gill damage and mortalities. In contrast, bithionol at 1 mg L^{-1} exhibited no morbidity yet was as effective as the current freshwater bath.

Bithionol toxicity and efficacy is dependent upon host species and target parasite. A concentration of 0.1 mg L^{-1} was reported as a possible therapeutic for the protozoan parasite, *Trichodina jadranica* in European eels, *Anguilla anguilla*, due to its capability to effectively treat entire recirculation systems, although it exhibited a relatively low therapeutic index (Madsen *et al.*, 2000). Conversely, Buchmann *et al.* (1992) demonstrated that bithionol at 0.1 mg L^{-1} was not efficacious as a static bath against gill parasitic monogeneans, *Pseudodactylogyrus* sp. and concentrations ≥ 1 mg L^{-1} were toxic, causing 100% mortality within 24 h in the European eel. Bithionol as a static bath has also been reported as effective in killing the ciliate *Tetrahymena pyriformis* at 60 mg L^{-1}, whilst being non-toxic to the minnow golden shiner, *Notemigonus crysoleucas* (Griffin, 1989).

For AGD-affected Atlantic salmon and rainbow trout, this study identified that a 1 h seawater bath using a bithionol concentration of 1 mg L^{-1} exhibited no morbidity and reduced amoeba numbers and percent lesioned gill filaments from the seawater control. Nonetheless, with the scale and intensity of salmonid farming occurring in Tasmania, a bithionol bath treatment would be impractical as it is

insoluble and would require a large amount of the compound to treat the bath water. On the other hand, with the determination of toxicity to the target animal and its efficacy with respect to AGD it would be possible to incorporate bithionol in-feed and assess as a treatment for the control of AGD in Atlantic salmon. Furthermore, with the increasing need for the use of chemotherapeutants in aquaculture, it is very important to minimise the accumulation of chemicals in food for human consumption and the effect on the environment. Producing an in-feed treatment would assist in alleviating the release of large amounts of the compound into the surrounding water (Findlay *et al.*, 2000).

In conclusion, bithionol was toxic above 25 mg L^{-1} in freshwater (municipal source, 15.5°C, pH 7) and seawater (35‰, 15.5°C, pH 8.2) for both Atlantic salmon and rainbow trout. It did not appear to acutely affect the gill toxicity parameters of SDH, Na^+/K^+ ATPase activity, or the plasma osmolality, chloride and no pathological changes in the gill or liver tissue were found over the 6 h period. Bithionol was efficacious as a 1 h seawater bath for treating amoebic gill disease caused by *Neoparamoeba* spp. reducing mean percent lesioned gill filaments and crude amoeba numbers compared to seawater control at all concentrations examined. Bithionol concentrations of 1, 5 and 10 mg L^{-1} produced an efficacy comparable to the currently used freshwater bath in Atlantic salmon. Bithionol warrants further investigation as a treatment for amoebic gill disease, including possible use as an oral medication.

3.6 Acknowledgements

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CHAPTER 4

EFFICACY OF BITHIONOL AS AN ORAL TREATMENT

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4 Efficacy of bithionol as an oral treatment for amoebic gill disease in Atlantic salmon, *Salmo salar*

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4.1 Abstract

This study examined the efficacy of bithionol as an oral treatment for Atlantic salmon, *S. salar* affected by amoebic gill disease (AGD). The current commercial management strategy of AGD is a costly 3 h freshwater bath. It is labour intensive and the number of baths needed appears to be increasing; hence there is an effort to identify alternative treatments. Efficacy was examined by feeding AGD-affected Atlantic salmon twice daily to satiation with bithionol, an antiprotozoal agent, at 25 mg kg^{-1} feed. Three seawater (35‰, 17°C) re-circulation systems were used, each consisting of three tanks containing 32 Atlantic salmon smolts with an mean (± SEM) mass of 90.4 g (± 5.2). Three feeds were examined in the trial including bithionol, plain commercial control and oil coated commercial control. Feeding commenced 2 weeks prior to exposure to *Neoparamoeba* spp. at 300 cells L^{-1} and continued for 28 days post exposure. Efficacy was determined by examining gross gill score and identifying percent lesioned gill filaments twice weekly for 4 weeks post exposure. Bithionol, when fed as a two week prophylactic treatment at 25 mg kg^{-1} feed, delayed the onset of AGD pathology and reduced the percent lesioned gill filaments by 53% and halved the gill score from two to one when compared with both the plain and oil controls during an experimental challenge. There were no palatability problems observed with mean feed intake of bithionol over the trial

duration with fish consuming higher levels of the bithionol diet compared to both the oil and plain controls. This study demonstrated that bithionol at 25 mg kg^{-1} feed, when fed as a two week prophylactic treatment for *Neoparamoeba* spp. exposure, delayed and reduced the intensity of AGD pathology, and warrants further investigation as an alternative to the current freshwater bath treatment for AGD-affected Atlantic salmon.

4.2 Introduction

Bithionol, a phenolic compound related structurally to hexachlorophene, has been used worldwide as a parasiticide for cattle, sheep, chickens, dogs and cats (Enzie and Colglazier, 1960). It is reported to have surfactant antimicrobial properties and thus is also effective against bacteria, moulds and yeast (Kim and Choi, 1998). It has been used as an alternative to praziquantel against human trematode and cestode infections, in particular paragonimiasis (Enzie and Colglazier, 1960; Takeuchi *et al.*, 1984), and has been widely used in veterinary medicine as it is active against flukes and cestodes (Mourot *et al.*, 1987). Bithionol has been found to stimulate lactic acid production, inhibit oxygen consumption and decrease glycolytic and oxidative metabolism in the human lung fluke, *Paragonimus westermani, in vitro* (Hamajima, 1973). Furthermore, it was postulated to be linked to a variety of processes including the inhibition of reduced nicotinamide adenine dinucleotide (NADH)-fumarate reductase and involved in protein phosphorylation (Reid *et al.*, 2001). With regards to fish, bithionol has been shown to be effective against the fish ciliates *Philasterides dicentrarchi* in turbot, *Scophthalmus maximus*, *Tetrahymena pyriformis in vitro* and *Trichodina jadranica* in European eels,

Anguilla anguilla, acting upon the mitochondrial respiratory chain and uncoupling electron transport (Griffin, 1989; Madsen *et al.*, 2000; Iglesias *et al.*, 2002).

Bithionol has been recorded as efficacious *in vitro* and *in vivo* when used as a bath treatment for salmonid parasites, including *Gyrodactylus* sp., *Ichthyobodo necator* (Santamarina *et al.*, 1991; Tojo *et al.*, 1994b) and *Neoparamoeba* spp. (Florent *et al.*, 2007a). Furthermore, due to bithionol being insoluble in water, it was advantageous to use bithionol as an oral medication, providing easy administration and limited fish handling (Tojo and Santamarina, 1998a). Bithionol at 40 g kg^{-1} feed was offered for 10 days at 2% body weight (BW) per day to rainbow trout, *Oncorhynchus mykiss*, infected with *Spironucleus salmonis*, *Gyrodactylus* sp. or *I. necator* and exhibited a reduction in parasitemia. Bithionol eliminated approximately 80% of *S. salmonis* from rainbow trout whilst both *Gyrodactylus* sp. and *I. necator* infections were reduced from a high to low intensity (Tojo and Santamarina, 1998a; b; c). Kim and Choi (1998) reported bithionol administered in-feed at 100-200 mg kg^{-1} BW significantly reduced the number of monogeneans *Microcotyle sebastis* on the gills of cultured rock fish, *Sebastes schlegeli*, with a 20 day feeding duration being most effective.

The protozoan parasite *Neoparamoeba* spp. is a free-living marine amphizoic amoeba and is believed to cause amoebic gill disease (AGD) (Kent *et al.*, 1988; Munday *et al.*, 1990; Adams and Nowak, 2004b). It attaches itself to the gills, particularly the secondary lamellae, and is characterised macroscopically by the presence of raised, white mucoid patches with histological presentation of single or

multi-focal epithelial hyperplasia leading to lamellar fusion (Adams and Nowak, 2001). It primarily affects salmonids and is a significant problem for the Atlantic salmon industry in Australia (Munday *et al.*, 2001; Nowak *et al.*, 2002). The current commercial treatment for AGD is freshwater bathing of affected fish, as it lowers gross gill lesions, mortalities and is environmentally friendly. However, it is labour and cost intensive as well as requiring fish handling, hence the need for effective oral medications (Parsons *et al.*, 2001a; Munday and Zilberg, 2003).

Several compounds have been examined for efficacy against AGD, including levamisole (Howard and Carson, 1995), chlorine dioxide, chloramine-T, hydrogen peroxide (Powell *et al.*, 2003; Powell and Clark, 2003), amprolium, albendazole, toltrazuril, and bithionol (Powell *et al.*, 2003). These compounds have been examined either as a bath treatment or in *in vitro* toxicity testing in seawater, with levamisole reported as lethal to *N. pemaquidensis* at concentrations $\geq$ 10 ppm *in vitro* (Howard and Carson, 1994), whilst chloramine-T at a concentration of 25 and 50 ppm reduced amoeba numbers equivalent to those seen in deionised water after 2 h (Powell and Clark, 2003). Amprolium was found to be an amoebastat at 1 mg L^{-1} and bithionol amoebicidal at 1 and 10 mg L^{-1} *in vitro* (Powell and Clark, 2003). Compounds that have been examined as in-feed treatment for AGD include the mucolytic compound L-cysteine ethyl ester (LCEE), which was reported to delay progression of pathology associated with AGD as well as reduce percent lesioned gill filaments by approximately 50% relative to the control when fed at 52.7 mg kg^{-1} fish d^{-1} for two weeks (Roberts and Powell, 2005). The purpose of the present study was to determine if bithionol, when administered orally as a prophylactic and

therapeutic treatment at 25 mg kg^{-1} feed to Atlantic salmon, could be used as an effective treatment against amoebic gill disease affected Atlantic salmon.

4.3 Materials and Methods

4.3.1 Fish husbandry and maintenance

Atlantic salmon (AS) diploid mixed-sex spring smolts, with a mass of 90.4 ± 5.2 g and a fork length of 21.1 ± 0.3 cm (N = 288) were obtained from SALTAS salmon hatchery (Tasmania, Australia). Fish were maintained at the University of Tasmania Aquaculture Centre for a minimum of three weeks prior to experimentation. Salmon were acclimated to seawater (30‰, 1 µm filtered, mean ± SEM temperature of 16 ± 0.8°C) over 14 days in one 3000 L Rathburn tank with recirculated water and an individual biofilter system. The tank received constant aeration and oxygen levels were monitored daily using a Handy Gamma Oxy Guard (Birkerød, Denmark), with dissolved oxygen levels of 94.0 ± 0.2% satuartion. Fish were fed a commercially available diet of 4 mm Atlantic Salmon Grower LE pellets (Skretting™, Hobart, Australia) twice daily to satiation throughout the seawater acclimation period.

4.3.2 Experimental design and challenge method

Two hundred and eighty-eight Atlantic salmon were equally and randomly allocated into nine tanks at a stocking density of 6.6 ± 0.1 g L^{-1}. The tanks were in three separate seawater re-circulation systems, each consisting of three 590 L tanks, a 500 L header, and a 500 L sump providing a total volume of 2770 L. All tanks received constant aeration with water temperature and oxygen levels monitored daily. Fish were acclimated from 30 to 35 ‰ over seven days and allowed to

habituate to the systems for the following seven days, with system conditions maintained on a photoperiod of 12 h each of light and dark, with a water temperature of 16.7 ± 0.3°C, pH 8.19 ± 0.01 and feeding of standard commercial feed prior to commencing experimental procedures. Following seven days conditioning, each system was randomly allocated a treatment. Treatments consisted of (1) commercial feed (control), (2) commercial feed coated with fish oil (control with oil) and (3) commercial feed coated with combination of 25 mg kg^{-1} feed bithionol and fish oil. This dose was chosen based on previous work, which showed bithionol to be toxic when administered as a bath treatment at concentrations > 10 mg L^{-1} (Florent *et al.*, 2007a). Fish were fed to satiation twice daily at 0830 h and 1630 h throughout the entire study with feed intake recorded daily. In order to determine tank feed intake an average pellet weight for each diet was obtained. Daily, the numbers of uneaten feed pellets were counted and multiplied by the average pellet weight, and feed intake was determined by subtracting the weight of uneaten feed from the feed placed into the tank. Feed intake was determined as a percent of the tank biomass by dividing the mean tank feed intake for the week by the tank biomass and multiplying by 100 to obtain the weekly mean feed intake as a percent of the tank weight. On Day 0 and 17, fish were only fed once due to weight check and sampling coinciding on these days. Weight checks with a minimum of ten fish per tank were conducted weekly to determine feed intake as percent body weight. All treatments were fed 14 days prior to exposure to *Neoparamoeba* spp. with feeding continued for a further 28 days after exposure.

Fish were experimentally exposed to *Neoparamoeba* spp. according to Morrison *et al.* (2004). Briefly, six donor Atlantic salmon were obtained from the University of Tasmania's Aquaculture Centre experimental AGD infection tank post-mortem. Gill baskets were excised from the salmon centrifuged in distilled water and rinsed with seawater three times to dislodge amoebae from the gills. Amoebae in seawater were allowed to adhere to Petri dishes for approximately 2 h at 18°C. Plates were washed with clean seawater and approximately 20 mL of seawater was added. Amoebae were allowed to adhere to Petri dishes overnight at 18°C. The adherent cells were removed by the addition of 1000 µL Hanks balanced salt solution with trypsin and EDTA (Appendix 1), washed, centrifuged and concentrated. Live amoeba counts were determined using a haemocytometer (Neubauer, BS 748). Three replicate counts were made with 18 large squares counted per replicate. The isolation obtained 2 500 000 amoeba delivering a final concentration of approximately 300 cells L^{-1} per system. Individual aliquots were placed into the three system sumps and foam fractionators were turned off.

4.3.3 Feed preparation

Commercially available 4 mm Atlantic Salmon Grower LE pellet (Skretting™, Hobart, Australia) was used for all treatments. Feed was prepared as needed in 100 g batches and stored at 4°C. The control with oil was prepared by moistening feed with 7 mL distilled water, then evenly coating with 5 mL of fish oil by shaking food in a plastic bag until well coated. The food was air dried for 24 h before being placed into an airtight container and held at 4°C. This same procedure was used to prepare the bithionol diet with the inclusion of 2.5 mg of bithionol (Sigma-Aldrich

Pty. Ltd, Castle Hill, Australia) which was combined with the fish oil prior to coating the moistened pellet.

4.3.4 Data Collection

Total tank mass was obtained weekly using a minimum of ten fish per tank. Fish were sampled (two fish per tank) at transfer, 14 days prior to infection and then twice weekly for 28 days. For each fish, mass and fork length data was recorded as well as gross and histological examination of internal organs for any signs of toxicity. The gross gill score data were recorded using categories seen in Table 4.3-1 (Adams and Nowak, 2003). Fish were anaesthetised or euthanized using clove oil at a concentration of 0.02% w/v and all procedures were conducted in accordance with the Australian code of practice for care and use of animals for scientific purposes under the guidance of the University of Tasmania Animal Ethics. Specific growth rate (SGR) and Fulton's condition factor (K) were calculated using equation 1 and 2. Fulton's condition factor was calculated using the individually sampled fish. The SGR was calculated using the average fish mass for each tank for Day -14 and Day 0 and then from Day 0 to the Day where there were a median number of 15 fish left. This was done due to the variation in fish numbers within tanks at the culmination of the trial.

$$SGR = \frac{\text{Ln weight (g)}_2 - \text{Ln weight (g)}_1}{\Delta \text{ time}} \times 100 \qquad [1]$$

$$K = \left[\frac{\text{weight(g)}}{\text{length} \times \text{length} \times \text{length (cm)}} \right] \times 100 \qquad [2]$$

Table 4.3-1. Scoring scheme for gross signs of amoebic gill disease on Atlantic salmon modified from Adams and Nowak (2003).

Infection Level	Score	Number of affected holobranchs
Clear	0	0
Light	1	< 2
Moderate	2	2-5
Heavy	3	> 5

4.3.5 Histology

The left gill basket was excised, rinsed gently in 0.2 µm filtered seawater, fixed in seawater Davidson's fixative and the liver, kidney, and muscle were fixed in 10% neutrally buffered formalin for 24 h, and then transferred to 70% ethanol. The second left anterior hemibranch was removed, along with a small section of liver, kidney and muscle, dehydrated, embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin and eosin (H & E). The sections were viewed under a light microscope (Olympus) at X100 to X400 magnifications. The number of filaments exhibiting AGD lesions were counted and expressed as proportions of the total number of filaments in each section (Parsons *et al.*, 2001a). A filament was counted only when the central venous sinus was visible in at least two-thirds of the filament and lamellae were of equal length bilaterally present to near the tip of the filament (Speare *et al.*, 1997).

4.3.6 Statistical Analysis

Statistical analysis was conducted using SPSS for Windows® (version 11.5). A mixed model analysis of variance (ANOVA) containing factorial and nested terms was used to determine differences with treatment and day sampled as fixed factors and tank as a random factor. There was no difference determined between the oil coated control and the plain control diets with respect to the AGD variables; therefore the control treatments were combined and compared to the bithionol treatment. The interaction between day sampled and treatment was examined first; if not significant, then treatment and day sampled could be examined individually. If a significant difference was identified among treatments and/or days sampled, then a Tukey's post hoc test was used to identify where the differences occurred. Homogeneity was determined using a residual plot and Levene's test. A result was considered significant if $p \leq 0.05$ and results are presented as a mean ± SEM. Relative percent reduction (RPR) was calculated for percent lesioned gill filaments using the following equation:

$$RPR = 1 - \left[\frac{\text{number of percent lesioned gill filaments in treated group}}{\text{number of percent lesioned gill filaments in control group}} \right] \times 100$$

4.4 Results

At the culmination of the trial all fish had exhibited signs of AGD. As expected, there were no significant differences between gross gill scores and percent lesioned gill filaments for the plain and oil control feeds; therefore they were combined. There appeared to be no problems associated with palatability as feed was eaten

across all treatments and mortalities were observed across all treatments. No toxicity problems were observed based on gross examination of internal organs and histological examination of the kidney, liver and muscle (histology not shown).

Bithionol treated fish exhibited significantly reduced lesioned gill filaments over the entire trial when compared to control fish (Fig 4.4-1a). Lesions were first observed on Day 7 in control groups compared to Day 10 in the bithionol group and percent lesioned gill filaments increased throughout the trial. Significant reductions in percent lesioned gill filaments were first observed between bithionol treated fish and control fish on Day 10 ($F_{2,15} = 50.101$, $p < 0.001$), and continued to the culmination of the trial at Day 28 ($F_{2,15} = 41.099$, $p < 0.001$) post-*Neoparamoeba* spp. exposure. Bithionol was successful in delaying the onset of percent lesioned gill filaments seen typically with AGD by 53% when compared to the control diets at the culmination of the trial (Day 28) post-exposure. A similar result was seen with the gross gill score, where on Day 28 controls had a mean gross gill score of two, which was significantly higher compared to bithionol treated fish exhibiting a mean gross gill score of one ($F_{2,15} = 25$, $p < 0.001$, Fig 4.4-1b). However, there were no differences observed in gross gill score on any other day throughout the trial.

There appeared to be no palatability problems associated with bithionol when fed at 25 mg kg^{-1} feed. Weekly feed intake in all treatment groups was determined significantly different over the duration of the trial ($F_{2,6} = 85.372$, $p = <0.001$) post-*Neoparamoeba* spp. exposure (Fig 4.4-2a). As the trial progressed the feed intake

began to decrease across all treatments particularly from Day 14 onwards for the control treatments and Day 21 onwards for the bithionol treatment, which coincided with heavier parasite load. However, there were no differences observed in the mean tank biomass mass of the fish across all treatments throughout the trial ($F_{2,6}$ = 0.348, p = 0.792 Fig 4.4-2b) or SGR ($F_{1,18}$ = 0.099, p < 0.001, Table 4.4-1) but a difference in K was observed ($F_{2,15}$ = 5.708, p = 0.014, Table 4.4-1).

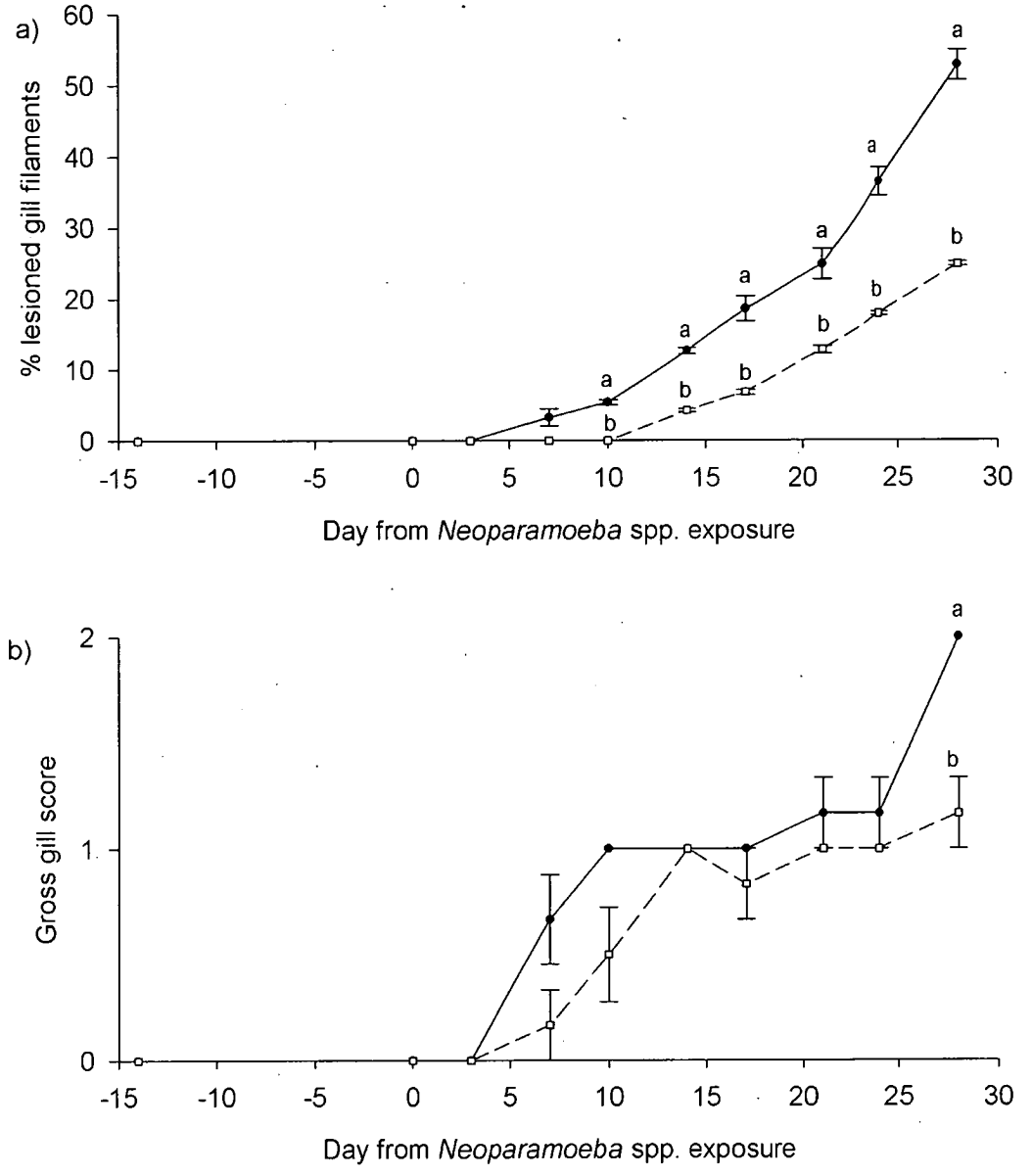

Fig 4.4-1. (a) percent lesioned gill filaments and (b) gross gill score for Atlantic salmon with amoebic gill disease (AGD) when fed either control feed (solid line) (n = 12) or bithionol at 25 mg kg^{-1} feed (broken line) (n = 6). No error bars indicate that all replicates exhibited the same value. Values are expressed as mean ± SEM. Letters denote significant differences among treatments at that sampling time ($p < 0.05$).

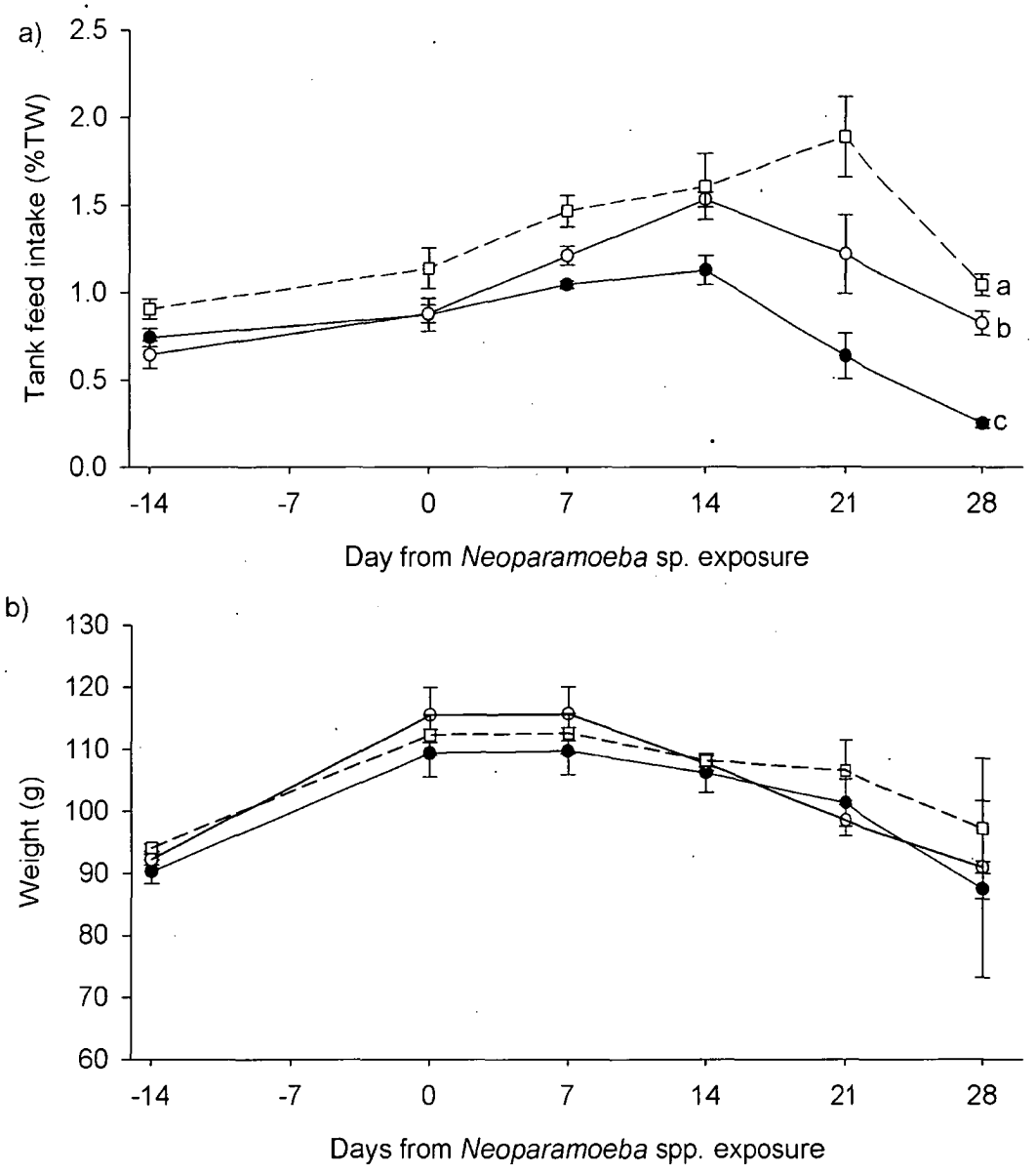

Fig 4.4-2. (a) weekly tank feed intake (n = 3) and (b) weekly tank biomass (n = 3) for Atlantic salmon exposed to *Neoparamoeba* spp. when fed either commercial feed (closed circles), oil-coated commercial feed (open circles) or bithionol at 25 mg kg^{-1} feed (broken line and open squares). Values are expressed as mean ± SEM. Letters denote significant differences among treatments over the trial duration ($p < 0.05$).

Table 4.4-1. Mean tank specific growth rate (SGR) (n = 3) and individual fish condition factor (K) (n = 6) for plain control, oil control and bithionol treated Atlantic salmon feed from Day -14 to 0 and Day 0 to Day 10 *Neoparamoeba* spp. exposure. Values are mean ± SEM. Asterisks indicate significant difference between treatments within each time point ($p<0.05$).

	Treatment					
	Day -14 to 0			Day 0 to Day 10		
	Plain	Oil	Bithionol	Plain	Oil	Bithionol
SGR (% day^{-1})	1.36	1.59	1.25	-0.11	-0.41	-0.12
	± 0.11	± 0.25	± 0.20	± 0.15	± 0.23	± 0.10
K (%)	0.89	0.81	0.84	1.04	0.97	1.16*
	± 0.07	± 0.04	± 0.01	± 0.04	± 0.04	± 0.02

4.5 Discussion

During a laboratory infection in which bithionol was orally administered at 25 mg kg^{-1} feed, the intensity of AGD pathology was significantly delayed and reduced compared to the control fish. A 53% relative reduction in percent lesioned gill filaments was observed when compared to the controls. Furthermore, gross gill scores were halved in the salmon treated with bithionol suggesting that this drug may be an effective therapeutic for AGD. This trial examined bithionol efficacy during the first four weeks of a *Neoparamoeba* spp. challenge and differences in gill score were only observed on the last day. Due to stocking density limitations, it was not possible to continue the trial further; however, it would be advantageous in future studies to continue past this time point. Similarly, bithionol was effective against the gill monogenean parasite *Microcotyle sebastis* infestations in marine rockfish, *Sebastes schlegeli*, resulting in a 66-93% reduction in parasite numbers when used at 1.25 g kg^{-1} feed for 10-20 days (Kim and Choi, 1998). In contrast, bithionol was reported to be ineffective as a treatment of *Hexamita salmonis*, *Gyrodactylus* sp., and *Ichthyobodo necator* infestations in rainbow trout, *Oncorhynchus mykiss* (Tojo and Santamarina, 1998a; b; c); however, classification of effectiveness in these experiments was complete elimination of the parasites. Bithionol when fed at 40 g kg^{-1} feed for 10 days did not achieve complete elimination of the parasites but produced an 80% reduction in *H. salmonis* whilst *Gyrodactylus* sp. and *I. necator* numbers were reduced from a high to low intensity. Whilst complete elimination of *Neoparamoeba* spp. would be welcomed, a treatment that is comparable in efficacy to bath treatment with freshwater and less labour intensive and more cost effective is a more realistic option, at least initially.

Bithionol has a melting point of 188°C; therefore it is possible that it could withstand the heat extrusion process so that it could be included at the feed preparation stage and, based upon the cost of bithionol, it would cost approximately \$120 tonne^{-1} (Sigma-Aldrich, 2006).

The standard laboratory-induced AGD infections using the isolation method described by Morrison *et al.* (2004) consistently induces AGD in Atlantic salmon. The severity of AGD in the laboratory is influenced by the amoeba challenge concentration and temperature (Zilberg *et al.*, 2001). Therefore, aggressive laboratory-induced AGD infections used in this study exhibit an increased rate of infection when compared to clinical AGD outbreaks on commercial salmon farms where there are less severe infection rates. Under laboratory conditions, there is no administration of freshwater baths and water is recirculated creating the potential for the continuous colonisation of the gills. Hence, differences observed between the two infection types are due to amoeba concentration and optimal laboratory conditions for AGD (Roberts and Powell, 2005). Fish affected with AGD on commercial farm field trials have been identified as having between 5% percent lesioned filaments coinciding with a freshwater bath and 10-20% 18 weeks post sea transfer (Adams and Nowak, 2003; Roberts and Powell, 2003a). This trial showed differences in the intensity of AGD pathology when fish were fed bithionol in an aggressive laboratory infection, warranting further investigation into the potential for oral administration of bithionol on commercial farms where AGD infections are typically chronic (Roberts and Powell, 2005). Further investigation of bithionol would include but not be limited to examining the pharmacokinetics and

bioavailability. The pharmacokinetics of bithionol regarding absorption, distribution, metabolism and excretion are not known within fish and are necessary for correct administration of bithionol.

Other compounds that have been examined as an in-feed treatment for AGD include the mucolytic drug L-cysteine ethyl ester (LCEE) (Roberts and Powell, 2005). Furthermore, the oral administration of nutritional supplements Aquacite™ and Betabec™ has been examined as an alternative treatment strategy (Mlynarski, *et al.,* unpublished data). It was identified that the progression of AGD when salmon were exposed to cohabitation-induced, aggressive laboratory infection was significantly delayed when LCEE was orally administered at 52.7 mg LCEE kg^{-1} fish d^{-1} over two weeks. Treated fish had approximately 50% less lesioned gill filaments than the control fish three days post-infection. However, there were palatability issues, in that the medicated feed intake was approximately 65% of the control feed (Roberts and Powell, 2005). Aquacite™ and Betabec™ nutritional supplements in a semi-commercial field trial were found to increase growth rate and maintain feed intake and conversion ratios when compared to control. Furthermore, the nutritional supplement exhibited a reduction in mortality and a delay in onset of AGD lesions (Mlynarski, *et al.,* unpublished data).

No signs of toxicity were observed when AGD-affected Atlantic salmon were fed bithionol at 25 mg kg^{-1} feed d^{-1} for 40 days. Similarly, no signs of toxicity were observed when bithionol was fed at 40 g kg^{-1} feed d^{-1} over 10 days to rainbow trout with either *H. salmonis*, *Gyrodactylus* sp., or *I. necator* infestations (Tojo and

Santamarina, 1998a; b; c). However, in contrast, bithionol exhibited signs of toxicity when used to treat *M. sebastis* infestations in marine rockfish at 1.25 g kg^{-1} feed d^{-1} for 10-20 days (Kim and Choi, 1998).

No problems were associated with the palatability of bithionol medicated feed, with mean bithionol consumption over the entire trial greater than both the oil and plain controls. As the trial progressed the feed intake observed across all treatments began to decrease particularly from Day 14 onwards for the control treatments and Day 21 onwards for the bithionol treatment, which coincides with heavier parasite load. There was however, no difference between treatments when examining specific growth rate, this could be due to the small sample size (n = 3) and the considerable amount of variation. In future studies it would be advantageous to examine SGR using average mass of individually sampled fish as opposed to tank average this may aid in comparing data. Conversely, fish fed bithionol exhibited a higher condition factor on Day 28 post exposure compared to both control treatments.

4.6 Conclusion

Bithionol exhibited potential as an oral treatment for AGD at 25 mg kg^{-1} feed when administered for two weeks prior to *Neoparamoeba* spp. exposure and 4 weeks following exposure. A reduction in percent lesioned gill filaments and gross gill score was achieved over the trial period. Furthermore, bithionol exhibited higher feed consumption throughout the study compared to plain and oil coated commercial diet and a reduction in mortalities. Further investigation of bithionol as

an in-feed treatment for AGD is warranted, including examining the effect of prophylactic and therapeutic treatments, running a similar experiment for a longer period of time to obtain more growth data, examining the effect of pulse feeding, or conducting trials under more realistic field conditions with lower exposure doses and freshwater baths.

4.7 Acknowledgements

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CHAPTER 5

FURTHER DEVELOPMENT OF BITHIONOL THERAPY AS A TREATMENT FOR AGD

In press with Journal of Fish Diseases

5 Further development of bithionol therapy as a treatment for AGD

Renée L. Florent, Joy A. Becker, Mark D. Powell.

5.1 Abstract

This study examined the efficacy of bithionol as a prophylactic or therapeutic oral treatment for Atlantic salmon, *Salmo salar*, affected by amoebic gill disease (AGD). Furthermore, it explored the interaction of bithionol oral therapy with the current standard treatment (a freshwater bath for at least 3 h). The efficacy of three feeds was determined in the trial by feeding AGD-affected Atlantic salmon at 1% body weight (BW) day^{-1} either oil coated commercial feed (control) or prophylactic and therapeutic bithionol at 25 mg kg^{-1} feed. Feeding commenced two weeks prior to exposure to *Neoparamoeba* spp. at 300 cells L^{-1} and continued for 49 days post exposure. Bithionol, when fed as a two week prophylactic or therapeutic treatment at 25 mg kg^{-1} feed, delayed the onset of AGD pathology and reduced the percentage of gill filaments with lesions. Administration of a 3 h freshwater bath at 28 days post-exposure significantly reduced amoebae numbers to a similar level across all treatments. In contrast, gross gill score and percent lesioned filaments were reduced to different extents, the control having a significantly higher score than both bithionol treatments. Following the freshwater bath, clinical signs of AGD increased at a similar level across all treatments, albeit controls were significantly higher than the bithionol treatments immediately following freshwater treatment. This study demonstrated that bithionol at 25 mg kg^{-1} feed, when fed as a two week

prophylactic or a therapeutic treatment, delayed and reduced the intensity of AGD pathology and warrants further investigation as a treatment for AGD-affected Atlantic salmon.

5.2 Introduction

Disease has numerous negative impacts on fish and aquaculture production, including reduced feed efficiency (Hedrick, 1998), impaired growth (Hedrick, 1998) and often death (Bakke and Harris, 1998). This in turn represents a direct loss of investment in feed, labour and stock to the farmer (Rábago-Castro *et al.*, 2006). When examining commercial farming of food fish, many bacterial, viral, fungal and parasitic pathogens have been recognised, not only in Tasmania but worldwide (Schmahl *et al.*, 1989; Stoffregen *et al.*, 1996). In the control of these pathogens, particularly external parasites of farmed marine fish, chemical treatments are common (Powell and Clark, 2004). However, since the turn of the 21st century there have been few new chemotherapeutic agents approved for use in aquaculture for several reasons, including concerns regarding environmental impact, cost and residues in food fish and the fact that it is often a limited market for drug companies (Shao, 2001). The continual improvement and development of cost-effective fish-husbandry practices, including efficacious chemotherapeutants, is critical for the success of the aquaculture industry and increased production (Stoffregen *et al.*, 1996).

Chemotherapeutants provide effective methods of preventing (prophylactic treatments) and controlling (therapeutic treatments) fish mortality and disease, and

are applied either via bath or oral administration (Armstrong, 1994; Howe *et al.*, 1999). Many fish farmers from North America (Thorburn and Moccia, 1993), Asia (Tonguthai, 1997), Europe (Alderman, 2002) and Africa (Hecht and Endemann, 1998) resort to the use of prophylactic treatments to aid in the prevention of epidemics. Several therapeutic agents have been examined for both prophylactic and therapeutic treatment of ectoparasitic infections; these include but are not limited to a formalin bath to treat trichodinads and monogeneans (Noga, 2000), chloramine-T for infections in salmon (Harris *et al.*, 2004), hydrogen peroxide for monogeneans (Mansell *et al.*, 2005), emamectin benzoate for sea lice (Ramstad *et al.*, 2002; Treasurer *et al.*, 2002), and bithionol as a bath treatment for salmonid parasites including *Gyrodactylus* sp., *Ichthyobodo necator* (Santamarina *et al.*, 1991; Tojo *et al.*, 1994b). More recently, bithionol has shown efficacy for the treatment of *Neoparamoeba* spp. (Florent *et al.*, 2007a; b).

Until recently the presumptive causative agents of AGD were thought to be two amphizoic amoebae *Neoparamoeba pemaquidensis* (Kent *et al.*, 1988; Roubal *et al.*, 1989) and *N. branchiphila* (Dyková *et al.*, 2005). However, attempts to determine the pathogenicity of either species by re-infecting fish using clonal, cultured, gill-derived strains have been universally unsuccessful (Kent *et al.*, 1988; Howard, 2001; Morrison *et al.*, 2005). *Neoparamoeba perurans* is a protozoan parasite that was recently described as the predominant aetiological agent of AGD of Atlantic salmon cultured in Tasmania, Australia (Young *et al.*, 2007). Amoebic gill disease has been diagnosed in a number of fish species cultured in the marine environment worldwide (Nowak *et al.*, 2002). It is a significant problem for

Australian salmon aquaculture with the disease accounting for 10-20% of the gross cost of production (Munday *et al.*, 2001; Nowak *et al.*, 2007). This is due not only to the cost of treating and managing the disease, but also to loss of fish condition and production costs. The mitigation of AGD in Tasmania is mostly due to freshwater bathing which was first recommended by Foster and Percival (1988). However, the efficacy is variable and has notably become increasingly less effective (Parsons *et al.*, 2001a; Powell and Clark, 2003); hence there is a push to identify alternative, more cost-effective and less labour intensive treatments, preferably delivered in-feed.

A large variety of antimicrobials, disinfectants and detergents have been examined both *in vitro* on isolated gill amoebae and *in vivo* as both bath and feed additives for AGD mitigation. Attempts to identify potential chemotherapeutic agents have been limited due to either lack of direct efficacy on the parasite, target fish toxicity or the cost of treatment (Alexander, 1991; Howard and Carson, 1994). Toxicity of several compounds to *Neoparamoeba* spp. has been examined *in vitro* including levamisole (Howard and Carson, 1995), chlorine dioxide, chloramine-T, hydrogen peroxide (Powell *et al.*, 2003; Powell and Clark, 2003), amprolium, albendazole, toltrazuril and bithionol (Powell *et al.*, 2003), all with low to moderate success. Howard and Carson (1994) reported that levamisole at concentrations $\geq$ 10 ppm *in vitro* were lethal to *N. pemaquidensis,* and chloramine-T concentrations of 25 and 50 ppm effectively reduced amoeba numbers after 2 h (Powell and Clark, 2003). Powell *et al.* (2003) found amprolium at 1 mg L^{-1} and bithionol at 1 and 10 mg L^{-1} to be amoebicidal *in vitro*. Compounds that have been examined as in-feed

treatment for AGD include the mucolytic compound L-cysteine ethyl ester (LCEE), which delayed progression of pathology associated with AGD and reduced percent lesioned gill filaments by approximately 50% relative to the control when fed at 52.7 mg kg^{-1} fish day^{-1} for 2 weeks (Roberts and Powell, 2005). However, it was not commercially viable and other mucolytic agents (N-acetyl cysteine) were not efficacious (Powell *et al.*, 2007). Bithionol, a phenolic compound reported as effective against bacteria, moulds and yeast (Kim and Choi, 1998), was found to be both non-toxic and efficacious; during a laboratory infection in which bithionol was prophylactically orally administered at 25 mg kg^{-1} feed, the intensity of AGD pathology was significantly delayed and reduced compared to the control fish (Florent *et al.*, 2007b).

The present study aimed to examine the interaction of bithionol oral therapy with the standard treatment (a freshwater bath for at least 3 h) and to examine the rate of re-infection. The role of alternative disease management strategies in controlling AGD is yet to be fully explored, but has the possibility to provide significant economic advantages to the industry. Currently, it is difficult to omit freshwater bathing and perhaps the best likelihood of success if through a multi-faceted approach to AGD management.

5.3 Materials and Methods

5.3.1 Fish husbandry and maintenance

Atlantic salmon, *Salmo salar*, (AS) diploid mixed-sex spring smolts, with a mass of 130.4 ± 9.8 g (N = 396) were obtained from SALTAS salmon hatchery (Wayatinah, Tasmania, Australia). Fish were maintained at the University of Tasmania Aquaculture Centre for a minimum of three weeks prior to experimentation. Salmon were acclimated to seawater (35‰, 1 μm filtered, temperature of 16.0 ± 0.8°C) over 14 days in one 3000 L Rathburn tank with recirculated water and an individual biofilter system. The tank received constant aeration and oxygen levels were monitored daily using a Handy Gamma Oxy Guard (Birkerød, Denmark), with dissolved oxygen levels of 92 ± 0.4% saturation. Fish were fed a commercially available 3 mm Atlantic Salmon Grower LE pellet (Skretting™, Hobart, Australia) twice daily to satiation throughout the seawater acclimation period.

5.3.2 Experimental design and challenge method

Three hundred and ninety-six Atlantic salmon were equally and randomly allocated into nine tanks at a stocking density of 9.9 ± 0.3 g L^{-1}. The tanks were in three separate ultraviolet light-treated seawater re-circulating systems, each consisting of three 590 L tanks, a 500 L header tank, and a 500 L sump providing a total volume of 2770 L. All tanks received constant aeration with water temperature and oxygen levels monitored daily. Fish were allowed to habituate to their respective systems for one week, with system conditions maintained on a

photoperiod of 12 h each of light and dark, with water temperature 17.0 ± 0.6 °C, and pH 8.08 ± 0.25, and were fed to satiation using a 3 mm commercial feed prior to commencing experimental procedures. Following conditioning, each tank was randomly allocated a treatment. The treatment groups were referred to as (1) the control, an oil coated commercial diet, (2) prophylactic, a bithionol treated diet at 25 mg kg^{-1} feed, fed for 14 days prior to exposure and (3) therapeutic bithionol fed at the presentation of clinical signs. This dose was chosen based on previous work, which showed bithionol to be effective when administered orally at 25 mg kg^{-1} feed (Florent *et al.*, 2007b). Fish were fed at a maximum of 1% body weight (BW) day^{-1} equally dispersed over 12 h throughout the entire study, with feed intake recorded daily. Daily feed intake was determined by subtracting the weight of uneaten feed from the amount of feed weighed out for that day. Weekly feed intake was determined as a percent of the fish biomass by dividing the average tank feed intake for the week by the average tank biomass and multiplying by 100 to obtain the weekly mean feed intake as a percent body weight. Weight checks of a minimum of half the fish per tank were conducted weekly to determine feed intake as percent body weight. All treatments were fed for 14 days prior to exposure to *Neoparamoeba* spp., with the control and therapeutic tanks receiving the control diet and feeding continued for a further 49 days post-exposure with respective treatments.

Fish were experimentally exposed to *Neoparamoeba* spp. which were isolated according to Morrison *et al.* (2004). Briefly, eight donor Atlantic salmon were obtained from the University of Tasmania Aquaculture Centre experimental AGD

infection tank. Gill baskets were excised from moribund salmon centrifuged at 400 *g* for 2 min in distilled water and rinsed with seawater three times to dislodge amoebae from the gills. Amoebae in seawater were allowed to adhere to Petri dishes for approximately 2 h at 18°C. Plates were washed with clean seawater and approximately 20 mL of seawater was added. Amoebae were allowed to adhere to Petri dishes overnight at 18°C. The adherent cells were removed by the addition of 1000 µL Hanks balanced salt solution with trypsin and EDTA (Appendix 1), washed, centrifuged at 400 *g* for 5 min and concentrated. Live amoeba counts were determined using a haemocytometer (Neubauer, BS 748). Three replicate counts were made with 18 large squares counted per replicate. The isolation delivered 2 500 000 amoeba at a final concentration of approximately 300 cells L^{-1} per system. Individual aliquots were placed into the three system sumps. The ultra-violet light units and foam fractionators were turned off immediately following exposure. Once gross lesions were observed and therapeutic treatment commenced all of the ultra-violet light units and foam fractionators were switched on and remained activated for the duration of the study.

5.3.3 Bath Administration

Following 28 days of exposure to *Neoparamoeba* spp., a 3 h freshwater (municipal source, 17.0°C, pH 7.2, treated with sodium thiosulphate at 0.005 mg L^{-1} to remove chlorine) bath was administered. Each tank of fish was placed into separate 100 L plastic containers at a stocking density of 30 g L^{-1}. Tubs received constant aeration with oxygen levels monitored every 10 min and maintained at 110% saturation using oxygen, if required. Temperature, salinity, pH, ammonia,

nitrate and nitrite were measured hourly for the duration of the bath. Following the 3 h bath fish were returned to their original tank.

5.3.4 Feed preparation

Commercially available 3 mm Atlantic Salmon Grower LE pellets (Skretting™, Hobart, Australia) were used for all treatments. Feed was prepared as needed in 1 kg batches and stored at 4°C. The control diet was prepared by moistening feed with 60 mL distilled water, then evenly coating with 30 mL of fish oil by shaking the feed in a plastic bag until well coated. The feed was air dried for 24 h on a tray in a force-draught fume hood (Forma Scientific, Ohio, USA), before being placed in an airtight container and stored at 4°C. This same procedure was used to prepare the bithionol diets with the inclusion of 25 mg of bithionol (Sigma-Aldrich Pty. Ltd, Castle Hill, Australia) which was combined with the fish oil prior to coating the moistened pellet.

5.3.5 Data Collection

Total tank mass of fish was obtained weekly, using a minimum of half the fish per tank. Fish were sampled (four fish per tank for 0-3 weeks post-exposure (PE) (n=12 per treatment) and three fish per tank from 4-7 weeks PE, (n=9 per treatment), 14 days prior to exposure, at the point of exposure, weekly for seven weeks and pre- and post-freshwater bath. For each fish, mass and fork length data were recorded, as well as gross examination of internal organs for any signs of toxicity. The gross gill score data were recorded using categories seen in Table 5.3-1(Adams and Nowak, 2003). Immediately prior to and following the freshwater bath treatment,

Neoparamoeba spp. were re-isolated from the gills using the technique of Florent *et al.* (2007a) modified from Howard and Carson (1995) and Powell and Clark (2003). Briefly, the right gill basket was excised and rinsed gently in 0.2 μm filtered seawater and individual arches were scraped with a bacterial spreader to remove mucus. Mucus was collected in individual 50 mL centrifuge tubes and re-suspended in sterile seawater up to 10 mL. A 100 μL aliquot of mucus-amoeba suspension was sampled and stained with 0.05% trypan blue viability stain at a dilution of 1:1. Live amoeba counts were determined using a haemocytometer. Three replicate counts were made with 18 large squares counted per replicate. The total number of live amoebae per fish was calculated and divided by the natural log of the fish mass to account for scaling differences in gill surface area with fish of different mass (Palzenberger and Pohla, 1992). In all cases, fish were euthanized or anaesthetised using clove oil at a concentration of 0.02% w/v. All procedures were conducted in accordance with the Australian Code of Practice for the care and use of animals for scientific research (7th edition), under administration of the University of Tasmania Animal Ethics Committee. Specific growth rates (SGR), Fulton's condition factor (K) and feed conversion ratio (FCR) were calculated using equation 1, 2 and 3, respectively. The K was calculated using the individual lethal fish samples and the SGR and FCR were calculated weekly using the average fish mass and average feed intake for each tank.

$$SGR = \frac{\text{Ln weight (g)}_2 - \text{Ln weight (g)}_1}{\Delta \text{ time}} \times 100 \qquad [1]$$

$$K = \left[\frac{\text{weight(g)}}{\text{length} \times \text{length} \times \text{length (cm)}} \right] \times 100 \quad [2]$$

$$FCR = \frac{\text{average feed intake (g)}}{\text{average fish biomass}_2\ \text{(g)} - \text{average fish biomass}_1\ \text{(g)}} \quad [3]$$

Table 5.3-1. Scoring scheme for gross signs of amoebic gill disease on Atlantic salmon modified from Adams and Nowak (2003).

Infection Level	Score	Number of affected holobranchs
Clear	0	0
Light	1	< 2
Moderate	2	2-5
Heavy	3	> 5

5.4 Histology

The left gill basket was excised, rinsed gently in 0.2 µm filtered seawater, fixed in seawater Davidson's fixative and the liver, kidney, and muscle were fixed in 10% neutrally buffered freshwater formalin for 24 h, and transferred to 70% ethanol. The second left anterior arch was removed, along with a small section of liver, kidney and muscle, dehydrated, embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin and eosin (H & E). The sections were viewed under a light

microscope (Olympus, Hamburg, Germany) at X100 to X400 magnifications. The number of filaments exhibiting AGD lesions were counted and expressed as proportions of the total number of filaments in each section. A filament was counted only when the central venous sinus was visible in at least two-thirds of the filament and the lamellae were of equal length bilaterally present to near the tip of the filament (Speare *et al.*, 1997). The size of the AGD lesions was recorded with lesion size determined by counting the number of hyperplastic interlamellar units within each lesion as described in Admas & Nowak (2001). A Leica DC300F digital camera (Leica Microsystems, Wetzlar, Germany) C-mounted to a light microscope (Olympus, Hamburg, Germany) was used for image capture. Liver, kidney and muscle tissue sections were examined for pathological changes including epithelial separation, aneurisms, change in pavement cells, chloride cells, hyperplasia, and inflammation (Mallatt, 1985; Takashima and Hibiya, 1994).

5.4.1 Statistical Analyses

Statistical analysis was conducted using SPSS for Windows® (version 11.5). A mixed model analysis of variance (ANOVA) containing factorial and nested terms was used to determine differences, with treatment and day sampled as fixed factor and tank as a random factor. The interaction between day sampled and treatment was examined first, and if this was not significant then treatment and day sampled were examined individually. If a significant difference was identified among treatments and/or days sampled then a Tukey's post hoc test was used to identify where the differences occurred. Differences among treatments of the tank variables, including feed intake, FCR and SGR, were analysed using a repeated measures

ANOVA, then a Tukey's post hoc test was used to identify where the differences occurred. Homogeneity was determined using a residual plot and Levene's test. A result was considered significant if $p \leq 0.05$ and results are presented as a mean ± SEM. Relative percent reduction (RPR) was calculated for percent lesioned gill filaments using equation 4.

$$RPR = 1 - \left[\frac{\text{number of percent lesioned gill filaments in treated group}}{\text{number of percent lesioned gill filaments in control group}} \right] \times 100 \quad [4]$$

5.5 Results

There were no differences observed in feed intake among treatments throughout the duration of the trial ($F_{2,6} = 0.793$, $p = 0.495$, Fig 5.5-1). As the trial progressed, the feed intake began to decrease across all treatments particularly from Day 14 onwards, which coincided with heavier parasite load. On Day -7 and 21 there was a sudden decrease in feed intake which was correlated with an increase in water temperature ($r = 0.359$, $n = 81$, $p = 0.001$). As expected, considering there were no differences between feed intakes, no differences in the mean condition factor (K) ($F_{2,6} = 0.209$, $p = 0.817$, Table 5.5-1) specific growth rate (SGR) ($F_{2,6} = 4.134$, $p = 0.0$, Table 5.5-1) or feed conversion ratio (FCR) ($F_{2,6} = 1.028$, $p = 0.413$, Table 5.5-1) were observed across all treatments throughout the trial.

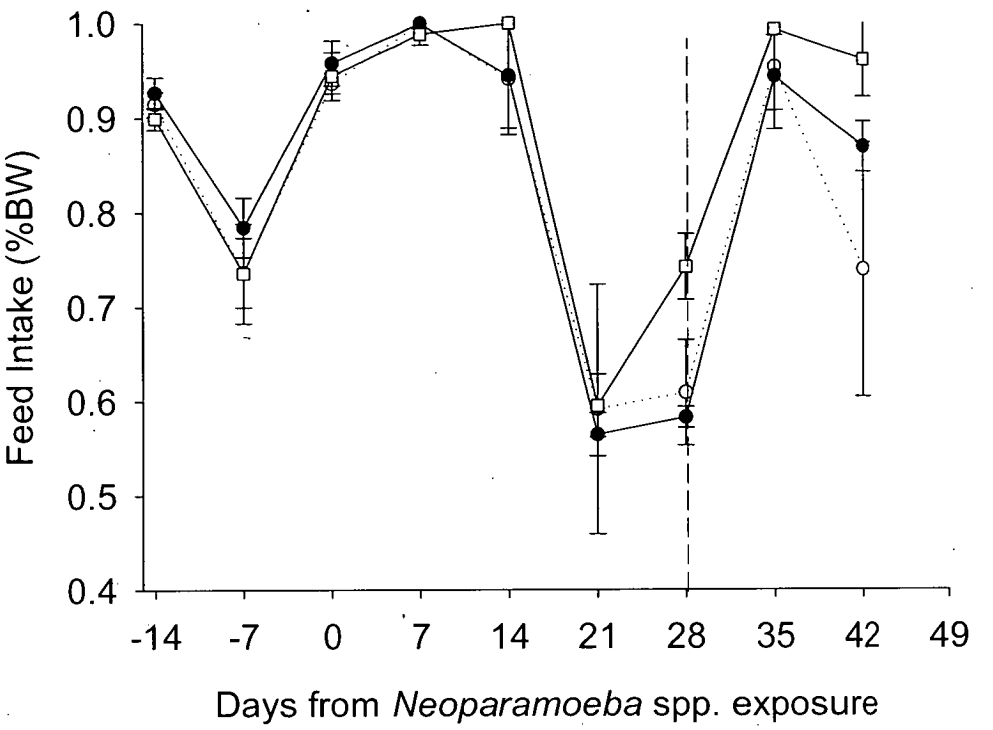

Fig 5.5-1. Mean (± SEM) weekly tank feed intake (n = 3) for Atlantic salmon exposed to *Neoparamoeba* spp. when fed either control feed (dotted line open circle), prophylactic bithionol at 25 mg kg^{-1} feed (solid line closed circle) or therapeutic bithionol at 25 mg kg^{-1} feed (solid line open square). No error bars indicate that all replicates within a treatment exhibited the same value. The vertical broken line indicates administration of a 3 h freshwater bath.

Table 5.5-1. Tank specific growth rate (SGR) (n = 3), individual fish condition factor (K) (n = 9) and tank feed conversion ratio (n = 3) for Atlantic salmon fed either oil coated commercial feed (C), prophylactic (PB) or therapeutic (TB) bithionol at 25 mg kg^{-1} feed from Day -14 to 0, Day 0 to 14, Day 14 to 28 and Day 28 to 42 from *Neoparamoeba* spp. exposure. All values are expressed as mean ± SEM.

	Treatment					
	Day -14 to 0			Day 0 to 14		
	C	PB	TB	C	PB	TB
SGR (% day^{-1})	1.14 ± 0.50	0.72 ± 0.08	0.53 ± 0.17	6.95 ± 3.12	10.18 ± 0.01	10.15 ± 0.07
K	0.86 ± 0.02	0.81 ± 0.04	0.85 ± 0.03	0.84 ± 0.03	0.87 ± 0.03	0.86 ± 0.03
FCR	0.19 ± 0.09	0.18 ± 0.02	0.30 ± 0.10	-0.02 ± 0.25	0.21 ± 0.01	0.44 ± 0.24
	Treatment					
	Day 14 to 28			Day 28 to 42		
	C	PB	TB	C	PB	TB
SGR (% day^{-1})	-1.21 ± 1.99	0.37 ± 0.29	-1.33 ± 1.19	1.77 ± 0.60	1.41 ± 0.12	2.57 ± 1.11
K	0.82 ± 0.04	0.82 ± 0.05	0.76 ± 0.04	0.80 ± 0.04	0.84 ± 0.04	0.77 ± 0.04
FCR	0.18 ± 0.11	5.63 ± 4.85	0.02 ± 0.12	0.13 ± 0.05	0.12 ± 0.01	0.11 ± 0.04

At the culmination of the trial all fish had exhibited signs of AGD. There appeared to be no problems associated with palatability as feed was eaten across all treatments and mortalities were observed across all treatments. No host toxicity was observed based on gross examination of internal organs and histological examination of the kidney, liver and muscle (histology not shown).

Fish administered bithionol at 25 mg kg^{-1} feed, either prophylactically (14 days prior to *Neoparamoeba* spp. exposure) or therapeutically (upon clinical signs of AGD), exhibited significantly reduced clinical signs of AGD including gross gill score ($F_{6,36} = 263.628$, $p < 0.001$, Fig 5.5-2a) and percent lesioned gill filaments over the entire trial, when compared to control fish ($F_{6,36} = 276.011$, $p < 0.001$, Fig 5.5-2b). Gross gill score and percent lesioned gill filaments were first observed on Day 7 in all treatment groups. From Day 14 onwards both the gross gill score and percent lesioned gill filaments increased at a greater rate in the control group compared to both bithionol treatments. Significant reductions in percent lesioned gill filaments were first observed between bithionol treated fish and control fish on Day 14 and continued throughout the duration of the trial ($F_{2,6} = 73.035$, $p < 0.001$, Fig 5.5-2b). Bithionol, when administered either prophylactically or therapeutically, was effective in reducing the proportion of lesioned gill filaments by approximately 30% when compared to the control treatment prior to the freshwater bath administered on Day 28 PE. At the culmination of the trial (Day 49 PE) the difference observed between both bithionol and control treatments was approximately 40% reduction in percent lesions. A similar result was seen with the gross gill score, where on Day 28 controls had a mean gross gill score of four. This

was significantly higher compared to bithionol treated fish, which exhibited a mean gross gill score of 2.8, and this difference continued throughout the trial ($F_{2,6}$ = 21.851, p = 0.002, Fig 5.5-2a). As expected, there were differences with respect to time for both gross gill score ($F_{6,36}$ = 263.628, p < 0.001, Fig 5.5-2a) and percent lesioned filaments ($F_{6,36}$ = 276.011, p < 0.001, Fig 5.5-2b) with values increasing until the freshwater bath was administered (Day 28 PE), after which values dropped to similar levels as seen on Day 14 PE and slowly began to increase until the culmination of the trial. Interestingly, the progression of lesion development was similar among all treatments following the freshwater bath until the culmination of the trial for both gross gill score and percent lesioned gill filaments (Fig 5.5-2a, b).

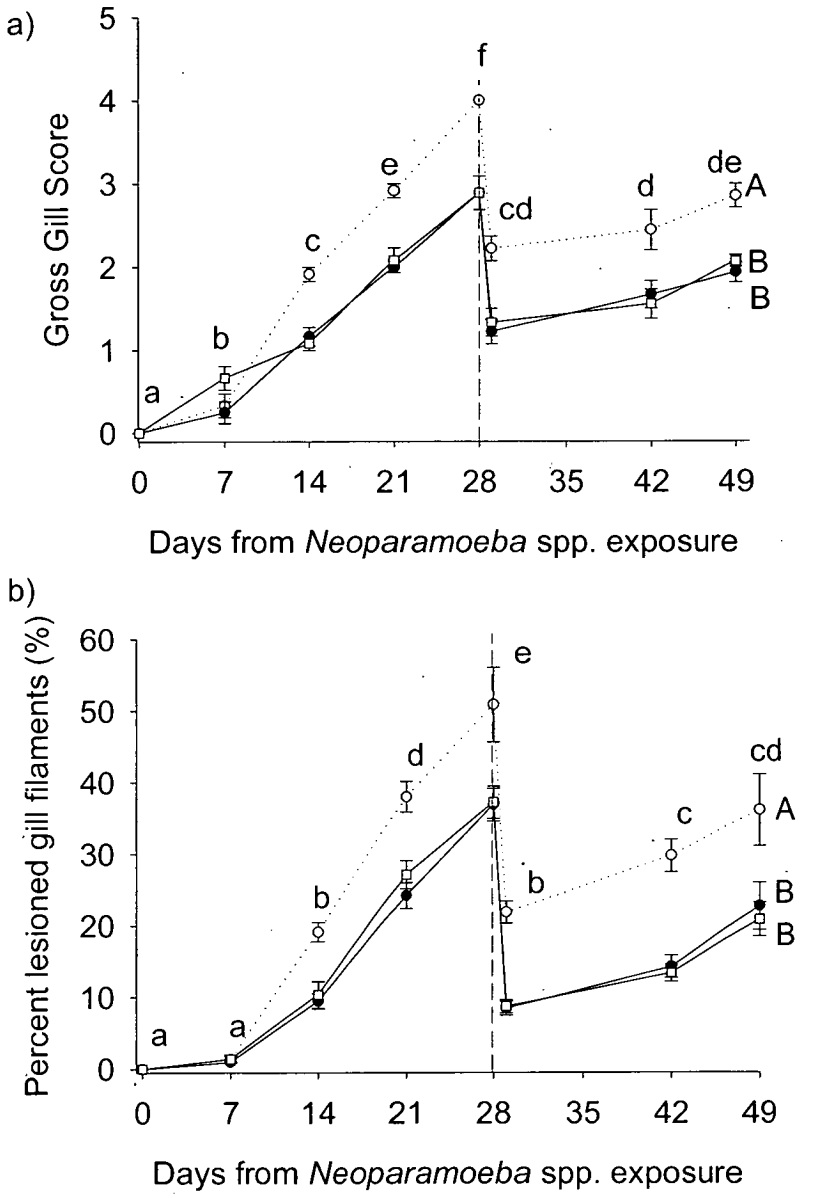

Fig 5.5-2. (a) gross gill score and (b) percent lesioned gill filaments for Atlantic salmon with amoebic gill disease (AGD) when fed either control feed (dotted line open circle), prophylactic bithionol at 25 mg kg^{-1} feed (solid line closed circle) or therapeutic bithionol at 25 mg kg^{-1} feed (solid line open square) (n = 12 pre-bath or 9 post-bath). All values are expressed as mean ± SEM. No error bars indicate that all replicates within a treatment exhibited the same value. The vertical broken line indicates administration of a 3 h freshwater bath. Lower and uppercase letters denote significant differences among days and treatments, respectively ($p < 0.05$).

When the size of the lesions was examined, both bithionol treatments had significantly smaller AGD lesions when compared to the control ($F_{2,6} = 7.442$, $p = 0.024$, Fig 5.5-3). A relative percent reduction in lesion size of approximately 20-30% was observed for both bithionol treatments from Day 21 onwards when compared to the control treatment. There was a significant difference with respect to time when examining lesion size ($F_{6,36} = 42.798$, $p < 0.001$, Fig 5.5-3), with lesion size increasing from Day 0 to Day 21 post-exposure following which it reached a plateau for all treatments until the culmination of the trial; interestingly lesion size did not decrease or increase following the administration of a 3 h freshwater bath.

Crude amoebae numbers were examined both pre- and post- freshwater bath administration with the control treatment exhibiting a greater number of amoebae compared to both bithionol treatments prior to the freshwater bath ($F_{2,24} = 11.130$, $p < 0.001$,Table 5.5-2). However, following the freshwater bath no difference was seen between treatments with crude amoebae numbers reduced to similar levels across all treatments ($F_{2,24} = 1.328$, $p = 0.284$, Table 5.5-2) which was supported by the proportional decrease in percent lesioned gill filaments (Fig 5.5-2b).

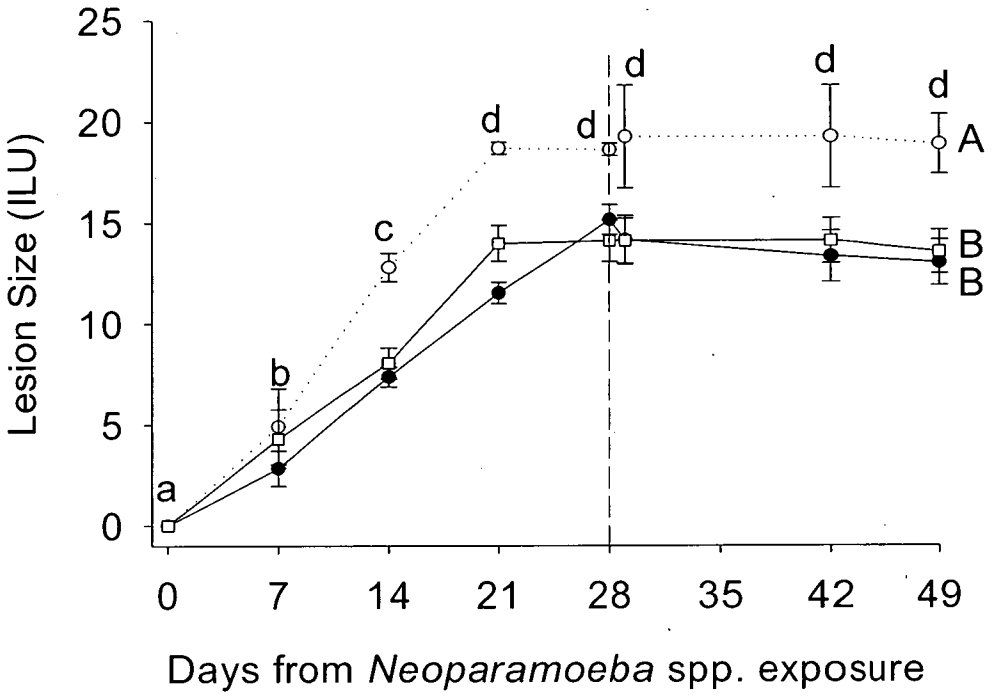

Fig 5.5-3. Lesion size in inter lamellae units (ILU) for Atlantic salmon with amoebic gill disease (AGD) when fed either control feed (dotted line open circle), prophylactic bithionol at 25 mg kg^{-1} feed (solid line closed circle) or therapeutic bithionol at 25 mg kg^{-1} feed (solid line open square) (n = 12 pre-bath or 9 post-bath). Values are expressed as mean ± SEM. No error bars indicate that all replicates within a treatment exhibited the same value. The vertical broken line indicates administration of a 3 h freshwater bath. Lower and uppercase letters denote significant differences among days and treatments, respectively ($p < 0.05$).

Table 5.5-2. Mean (± SEM) crude amoeba numbers and relative percent reduction pre- and post- 3 h freshwater bath for Atlantic salmon with amoebic gill disease (AGD) when fed either control feed, prophylactic bithionol at 25 mg kg^{-1} feed or therapeutic bithionol at 25 mg kg^{-1} feed. Letters superscripts denote significant differences among treatments ($p < 0.05$) ($n = 9$).

	Treatment		
	Control	Prophylactic bithionol	Therapeutic bithionol
Pre-bath amoeba numbers	228 588 ± 14 638[a]	149 017 ± 11 168[b]	168 629 ± 11 144[b]
		34.81%	26.23%
Post-bath amoeba numbers	5 259 ± 838[c]	3 733 ± 422[c]	4 423 ± 623[c]

5.6 Discussion

Atlantic salmon when fed bithionol at 25 mg kg^{-1} feed either as a two week prophylactic or a therapeutic treatment exhibited significantly delayed and reduced clinical signs of AGD compared to controls during a laboratory infection. Following 14 days post-exposure, both groups of treated fish had significantly less affected filaments than control fish. Prior to the freshwater bath administered on Day 28 PE this difference had decreased; however, there were still significantly fewer filaments affected than the control fish. A similar result was also seen with gross gill score where a difference of approximately one gill score between medicated fish and control fish from Day 14 PE onwards. Similarly, bithionol was reported effective as a 14 day prophylactic treatment for AGD in Atlantic salmon with gross gill score halve and a 53% reduction in percent lesions over 28 days (Florent *et al.*, 2007b). Bithionol was effective against the gill monogenean parasite *Microcotyle sebastis* infestations in marine rockfish, *Sebastes schlegeli*, resulting in a 66–93% reduction in parasite numbers when used at 1.25 g kg^{-1} feed for 10–20 days (Kim and Choi, 1998). In contrast, bithionol was reported to be ineffective as a treatment of *S. salmonis, Gyrodactylus* sp. and *I. necator* infestations in rainbow trout (Tojo and Santamarina, 1998a; b; c); however, in these studies effectiveness was defined as a complete elimination of the parasites. Bithionol when fed at 40 g kg^{-1} feed for 10 days did not achieve complete elimination of the parasites but produced an 80% reduction in *S. salmonis* while *Gyrodactylus* sp. and *I. necator* numbers were reduced from a high to low intensity (Tojo and Santamarina, 1998a; b; c).

Crude numbers of viable amoeba pre- and post-bath were found to be higher in the control fish compared to the medicated fish pre-bath; however, following the 3 h freshwater bath amoeba numbers were reduced to similar levels across all treatments with all exhibiting at least a 90% reduction which is similar to levels seen in previous studies (Parsons *et al.*, 2001a; Clark *et al.*, 2003). This was not seen with gross gill score and percent lesioned filaments which were knocked down proportionally rather than to a similar level. However, all treatments had a similar percent reduction in lesioned filaments of 60-70% following a freshwater bath and gill score was reduced from four to two for the controls and from three to one for both bithionol treatments.

The frequency of freshwater bathing conducted by the Tasmanian Atlantic salmon industry is determined by the gross examination of gills. Generally, the trigger for initiating a freshwater bath is a gross gill score of two or approximately 25% lesioned filaments. So in this study control fish were ready for their first freshwater bath around Day 14 PE whereas medicated fish were not at bath level until a week later. Additionally, freshwater bathing only removed approximately two thirds of the amoebae (Clark *et al.*, 2003), leaving one third of the parasites as a chronic persistent infection, which eventually triggers another freshwater bath and the cycle is repeated. Nowak *et al.* (2007) reported that re-infection following a freshwater bath occurred although lesions examined histologically immediately post-bath exhibited no trophozoites of *Neoparamoeba* spp. However, several studies have shown that reinfection is possible post bath without adding fresh amoebae to the seawater (Adams and Nowak, 2001; 2004a; Gross *et al.*, 2004). Hence, following

the freshwater bath at Day 28 PE control fish were ready for a second bath at Day 35 PE compared to medicated feed where bath level was not reached until Day 49 PE. This indicated that it may be advantageous to use bithionol as a combination therapy with the current freshwater mitigation to achieve the best results for reduction in clinical signs of AGD.

Not only did medicated fish exhibit reduced clinical signs of AGD throughout the study but the size of AGD lesions was reduced significantly compared to control fish. Lesion size seemed to increase until Day 21 after which it reached a plateau for all treatments. Interestingly, even following a freshwater bath, lesion size remained the same for all treatments. There was a larger variability in lesion size following the freshwater bath indicating that lesion size could be altered following the bath. However, this could be due to larger lesions reducing in size slightly, whilst smaller lesions had a greater rate of reduction. Furthermore, percent lesions decreased, creating the possibility that the freshwater bath removed smaller lesions whilst the larger lesions remain. Further investigation is warranted to investigate the relationship between lesion size and the impact of a freshwater bath. There has been minimal published information regarding AGD lesion size. Adams and Nowak (2001) examined lesion size in order to describe the distribution and structure of AGD lesions and Embar-Gopinath *et al.* (2005; 2006) examined lesion size with respect to the presence of different salmonoid gill bacteria. Lesion size in this study was similar to that in Embar-Gopinath *et al.* (2006) but larger than in Embar-Gopinath *et al.* (2005).

There were no differences in feed intake with fish in all treatment groups eating a similar amount throughout the duration of the study. As the trial progressed the feed intake observed across all treatments began to decrease, particularly from Day 14 onwards, which coincides with heavier parasite load. Furthermore, on Day -7 and 21 there was a sudden decrease in feed intake. This difference coincided with a sudden increase in water temperature, although this was still within the physiological range of Atlantic salmon. This is in contrast to previous studies where intake of bithionol-treated feed was significantly higher than of control feeds (Florent *et al.*, 2007b). This study identified no differences between treatments when examining SGR, K and FCR which is similar to previous studies with the exception of condition factor where fish fed bithionol at 25 mg kg^{-1} feed to satiation twice daily for 28 days following *Neoparamoeba* spp. exposure exhibited a higher condition factor than control groups (Florent *et al.*, 2007b). It is possible that the difference seen between these two studies was due to the different method of feeding that is either to satiation twice daily or at 1% BW day^{-1}. However, fish in this study did not to reach the 1% limit regularly, suggesting that there could be another factor involved such as time of year or temperature. Further investigations would be needed to determine this.

Bithionol exhibited potential as an oral treatment for AGD at 25 mg kg^{-1} feed when administered either for 2 weeks prior to (prophylactic) *Neoparamoeba* spp. exposure or at clinical signs of AGD (therapeutic). A reduction in percent lesioned gill filaments, lesion size, and gross gill score was achieved over the trial period. Bithionol warrants further investigation as a possible in-feed treatment for AGD in

Atlantic salmon, especially as a combination therapy with the standard freshwater bath treatment. Also, examining the effect of pulse feeding or conducting trials under more realistic field conditions with lower exposure doses and freshwater baths would be of interest.

5.7 Acknowledgements

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CHAPTER 6

GENERAL DISCUSSION

6 General Discussion

Throughout this thesis it has be shown that bithionol is toxic to *Neoparamoeba* spp. *in vitro* and significantly delayed and reduced the intensity of amoebic gill disease (AGD) pathology when administered as either a bath or oral treatment. Therefore, revealing the potential of using bithionol as either an alternative treatment for AGD or as a combination therapy with the current commercial freshwater treatment. This indicated that bithionol warrants further investigation as a treatment for AGD. The primary aim of this research described in previous chapters was to identify an improved method of treatment for AGD caused by *Neoparamoeba* spp. The improved method would have an enhanced efficacy, alleviating clinical signs and associated pathology compared to the current mitigation strategy of freshwater bathing whilst being economical. This research focused primarily on development of an oral treatment for AGD, as producing an in-feed treatment would assist in alleviating the release of large amounts of the compound into the surrounding water (Findlay *et al.*, 2000) and is less labour intensive than bathing fish. The research documented in this thesis successfully identified possible improvements or alternative treatments for AGD, involving the oral administration of bithionol.

Herein will discuss the major findings related to the improved or alternative AGD treatments, address the economic viability and touch on some environmental issues of using such treatments. These past results have been not only necessary for the formation of this thesis but have greatly advanced the knowledge surrounding the

treatment of AGD caused by *Neoparamoeba* spp. and the use of bithionol as a treatment for parasites. Most notably, the development of a three-tiered approach to identifying drug treatments: Stage I of drug screening would be the development of single- and multi-day *in vitro* toxicity assays and host toxicity procedures; Stage II of testing, whereby animals are offered drug-coated feed and subsequently experimentally challenged with the parasite to determine fish toxicity and efficacy for preventing clinical signs of disease; and Stage III testing involving field trials. The research in this thesis has laid the necessary ground work for bithionol and bithionol sulphoxide to be progressed to the final stages of treatment investigation with a clinical trial.

A similar drug model has been used to develop the current in-feed treatment for sea lice emamectin benzoate as a 0.2 % aquaculture pre-mix identified as Slice®. A great deal of effort has gone into the development of sea lice treatments, including bath or dip treatments using dichlorovos, trichlorfon, azamethiphos, cypermethrin, carbaryl, pyrethroids and hydrogen peroxide with varying degrees of efficacy (Costello, 1993; Roth *et al.*, 1993). Oral doses of ivermectin (Palmer *et al.*, 1987), insect growth regulators (Roth *et al.*, 1993) and emamectin benzoate (Stone *et al.*, 2000) have also been examined. Some of which made it to commercial field trials whilst others only to the small scale experimental stages.

The efficacy and toxicity of SLICE® (emamectin benzoate) as a treatment for sea lice infestations on Atlantic salmon was evaluated through an extensive series of clinical studies and field trials (reviewed in Johnson and Margolis, 1993; Roth *et*

al., 1993; MacKinnon, 1997). Initially, emamectin benzoate was used in an unformulated state and incorporated in an edible oil for application as a coating on pelleted feed. Dose rate determination and dose rate confirmation studies were conducted at several locations throughout Scotland. Small field trials were conducted in Scotland, Norway, Canada, and Chile. In these field trials, fish were fed a commercial feed treated with SLICE®, 0.2% emamectin benzoate aquaculture premix. A daily diet of medicated feed was administered at the recommended dose rate of 50 $\mu g\ kg^{-1}$ biomass day^{-1} for 7 consecutive days (Roth *et al.*, 1993; MacKinnon, 1997). Commercial field trials were conducted in Canada, Chile, Scotland, and Norway. In Norway the efficacy of SLICE® was compared to that of another in feed treatment, teflubenzuron, a chitin synthesis inhibitor known commercially as Ektobann®. Results from four study sites in Norway showed SLICE® medicated feed provided better sustained efficacy against sea lice when compared to teflubenzuron-treated diets (MacKinnon, 1997). Extensive studies have also been conducted examining the effects on the host, the environmental impacts and the pharmacokinetics of SLICE® (MacKinnon, 1993; Chukwudebe *et al.*, 1996; Mushtaq *et al.*, 1996), this enabled the drug emamectin benzoate to licensed for use in food fish under the registered trademark of SLICE®.

Variability in the effectiveness of freshwater bathing is suggested to be attributed to water chemistry, in particular total hardness (mg L^{-1} $CaCO_3$) (Parsons *et al.*, 2001a; Parsons *et al.*, 2001b; Clark, 2002; Powell and Clark, 2003; Roberts and Powell, 2003a). Freshwater bathing is an environmentally friendly treatment and aids in maintaining the 'clean-green' image of the Australian salmon industry.

Roberts and Powell (2003a) reported that in using soft freshwater (19.3 - 37.4 mg L^{-1} $CaCO_3$) delays reinfection and subsequent pathology by at least two weeks compared to hard freshwater. Providing the ability to reduce the annual number of bath from ten to seven baths per year (Mitchell, 2001; Roberts, 2004) equating to a saving of approximately $9.9 million annually. It is possible that with the combined use of soft freshwater and an in-feed treatment that the freshwater bath frequency could be reduced even further, thus saving more time and money.

A considerable amount of effort has been placed on identifying possible treatments for AGD with varying success. Attempts to identify potential chemotherapeutic agents have been limited due to either lack of direct efficacy on the parasite, target fish toxicity or the cost of treatment (Alexander, 1991; Howard and Carson, 1994). The above model has allowed for a strategic system to screen and identify candidate drugs from a large pharmacopeia, whilst maintaining effective resource management. Toxicity of several compounds to *Neoparamoeba* spp. has been examined *in vitro* including levamisole (Howard and Carson, 1995), chlorine dioxide, chloramine-T, hydrogen peroxide (Powell *et al.*, 2003; Powell and Clark, 2003), amprolium, albendazole, toltrazuril and bithionol (Powell *et al.*, 2003), all with low to moderate success. Howard and Carson (1994) reported that levamisole at concentrations $\geq$ 10 ppm *in vitro* were lethal to *N. pemaquidensis,* and chloramine-T concentrations of 25 and 50 ppm effectively reduced amoeba numbers after 2 h (Powell and Clark, 2003).

Using Stage I of the drug model, it was identified that bithionol was amoebicidal at 1 and 10 mg L^{-1} (Powell *et al.*, 2003); this was examined further using a greater variety of concentrations ranging from 0.1 to 10 mg L^{-1} (Chapter 2). Bithionol and bithionol sulphoxide (a cheaper and supposedly less toxic alternative) were successful at reducing amoeba numbers relative to seawater controls. Both were toxic to *Neoparamoeba* spp. at concentrations ranging from 0.1 to 10 mg L^{-1} over 72 h. However, freshwater still remained the most toxic with complete mortality of *Neoparamoeba* spp. seen at 48 h. Bithionol was reported to be toxic to the trophozoites of the protozoan parasite *Giardia lambia* at 0.42 and 0.28 mM over 24 and 72 h, respectively (Takeuchi *et al.*, 1985). Similar results were seen with respect to the human protozoan parasite *Trichomonas vaginalis* where bithionol was toxic at 0.42 mM after 24 h (Takeuchi *et al.*, 1985). The toxicity of bithionol *in vitro* was also examined using the human and primate protozoan parasite *Entamoeba histolytica* and found to kill virtually all axenic and polyxenic amoeba in 24 h at both 0.42 and 0.28 mM (Takeuchi *et al.*, 1984).

With regards to *Neoparamoeba* spp. very few drugs to date have been successful at Stage I and thus moved onto Stage II, where animals were offered drug-coated feed and subsequently experimentally challenged with the parasite to determine fish toxicity and efficacy for preventing clinical signs of disease. Within this thesis, Atlantic salmon, *Salmo salar*, and rainbow trout, *Oncorhynchus mykiss*, were either administered bithionol as a bath treatment or offered bithionol-coated feed and subsequently experimentally challenged with *Neoparamoeba* spp. to determine fish toxicity and efficacy for preventing clinical signs of AGD. An efficient way to

examine initial *in vivo* efficacy and toxicity is through bath administration of the compound as it requires less resources and time. For AGD-affected Atlantic salmon and rainbow trout, it was identified that a 1 h seawater bath using a bithionol concentration of 1 mg L^{-1} exhibited no morbidity and reduced amoeba numbers and percent lesioned gill filaments from the seawater control. Bath administration of bithionol has been examined in numerous species infected with various parasites. Bithionol toxicity and efficacy is dependent upon host species and target parasite. With regard to salmonid parasites, such as *Gyrodactylus* sp. and *Ichthyobodo necator*, Santamarina *et al.* (1991) observed limited toxicity and complete *in vitro* efficacy against *Gyrodactylus* sp. in rainbow trout at 12.5 mg L^{-1}, with a minimum 20 mg L^{-1} reported as efficacious *in vivo*. Tojo *et al.* (1994b) stated that bithionol was efficacious *in vivo* against *I. necator* in rainbow trout at 25 mg L^{-1} for a 3 h bath on two consecutive days; higher concentrations exhibited some mortality. Bithionol was reported as efficacious against *Neoparamoeba* spp. at concentrations ranging from 1 to 35 mg L^{-1}; however, at concentrations $\geq$ 10 mg L^{-1} high percentage mortality was seen within 3 h for both Atlantic salmon and rainbow trout (Florent *et al.*, 2007a). Finally, Madsen *et al.* (2000) determined that bithionol at 0.1 mg L^{-1} was an effective treatment against trichodiniasis in European eels, *Anguilla anguilla*, but found bithionol to have a relatively narrow therapeutic index. Conversely, Buchmann et al. (1992) demonstrated that bithionol at 0.1 mg L^{-1} was not efficacious as a static bath against gill parasitic monogeneans, *Pseudodactylogyrus* sp. and concentrations $\geq$ 1 mg L^{-1} were toxic causing 100% mortality within 24 h in the European eel. Bithionol as a static bath has also been reported as effective in killing the ciliate *Tetrahymena pyriformis* at 60 mg L^{-1},

whilst being non-toxic to the minnow golden shiner, *Notemigonus crysoleucas* (Griffin, 1989). Nonetheless, with the scale and intensity of salmonid farming occurring worldwide, a bithionol bath treatment would be impractical, as it is insoluble and would require a large amount of the compound to treat the bath water.

On the other hand, with the determination of toxicity to the target animal and its efficacy with respect to AGD it would be possible to incorporate bithionol in-feed and assess as an oral treatment for the control of AGD in Atlantic salmon. Although, *Neoparamoeba* spp. is an external parasite, similar to the treatment strategies for sea lice, the desired delivery method, from an industry perspective is an in-feed drug therapy. Reasons for this included the fact that in-feed treatment allows medication during adverse weather conditions and on exposed sites and reduces the chance of cross-infestation that may occur during the several days necessary to apply bath treatments to all cages on a site because an in-feed treatment permits simultaneous medication of all cages on a site. Hence the treatment of sea lice with an emamectin benzoate as a 0.2 % aquaculture pre-mix identified as SLICE® is ideal for famers compared to the bath treatments previously used (Stone *et al.*, 1999). Furthermore, with the increasing need for the use of chemotherapeutants in aquaculture, it is very important to minimise the accumulation of chemicals in food for human consumption and the effect on the environment. Therefore, consideration for residues in the animal and accumulations and the effect on the environment must be taken when examining any compounds for use as treatments for disease in aquatic animals.

Bithionol has been suggested as an oral medication due to its ability to provide easy administration and limited fish handling (Tojo and Santamarina, 1998a). Bithionol has a wide spread use in the treatment of numerous parasites in different hosts (El-Sayad, 1997). In cattle, bithionol sulphoxide was reported as effective at reducing *Fasciola hepatica* in the rumen when administered a single dose at 30 mg kg^{-1} for the first four weeks but numbers returned to pre treatment levels 20 weeks following treatment (Ueno *et al.*, 1973). However, when administered as a single dose at 90 mg kg^{-1} bithionol sulphoxide reduced the natural rumen fluke infestation of cattle by 70% (Prasittirat *et al.*, 1997). Bithionol was reported as effective in removing the worms *Thysanosoma actinioides* (Allen *et al.*, 1962) and *Hymenolepris nana* (Maki and Yanagisawa, 1985) from sheep and mice, respectively. With examining aquatic animal health, bithionol at 40 g kg^{-1} feed was offered for 10 days at 2% body weight (BW) per day to rainbow trout infected with *Spironucleus salmonis* (formerly *Hexamita salmonis*), *Gyrodactylus* sp. or *I. necator* and exhibited a reduction in parasite load. Bithionol eliminated approximately 80% of *S. salmonis* from rainbow trout whilst both *Gyrodactylus* sp. and *I. necator* infections were reduced from a high to low intensity (Tojo and Santamarina, 1998a; b; c). Kim and Choi (1998) reported bithionol administered in-feed at 100-200 mg kg^{-1} BW significantly reduced the number of monogeneans *Microcotyle sebastis* on the gills of cultured rock fish (*Sebastes schlegeli*), with a 20 day feeding duration being most effective. Furthermore, bithionol was reported as efficacious delay and reducing clinical signs of AGD in Atlantic salmon when fed at 25 mg kg^{-1} feed either as a two week prophylactic treatment or a therapeutic treatment (Florent *et al.*, 2007b).

Bithionol was successful in delaying and reducing clinical signs of AGD by approximately two weeks and even reducing the amoebae numbers by half would theoretically half the number of baths. Bithionol incorporated into feed would cost approximately \$150 tonne^{-1} (Sigma-Aldrich, 2006); if this successfully halved the bath numbers it has the potential to lessen the cost of treatment compared to freshwater. When examining the cost of bithionol and bithionol sulphoxide the price difference is considerable with bithionol eight times more expensive than bithionol sulphoxide, thus bithionol sulphoxide if as effective as bithionol is a significantly cheaper alternative.

Bithionol has been identified as successful in stage I and II of the three-tiered approach mentioned above. In order to continue through to stage III involving field testing it would be advantageous to examine the pharmacology and residue levels of bithionol and its metabolites. Previous studies show that bithionol may work more effectively when high infection pressure is observed such as that in a laboratory trial as opposed to a low infection pressure seen typically in a farm situation (Chapter 5). Thus examining the efficacy of both bithionol and bithionol sulphoxide at lower infections pressure would be beneficial and aid in collating data to apply for an experimental license.

The next phase of the research would include further investigation of bithionol as an in-feed treatment for AGD, incorporating examination of the effect of prophylactic and therapeutic treatments running experiments for a longer period of time to obtain more growth data, examining the effect of pulse feeding, or

conducting trials under more realistic field conditions with lower exposure doses and freshwater baths. Furthermore, sophisticated studies on the pharmacology of possible treatments are often lacking, owing in part to the limited knowledge about the biochemistry of parasite, which are more difficult to study *in vitro* than bacteria (Barrett-Connor, 1982). Therefore, in addition to studying clinical efficacy of bithionol as a treatment for AGD, pharmacokinetic examination is essential to establish correct dosage regimes and hence optimal drug usage (Samuelsen and Ervik, 1999; Haug and Hals, 2000; Stringer, 2001; Birkett, 2002; Katharios *et al.*, 2002). Pharmacokinetics describes the relationship between the dose and the unbound drug concentration at a drug receptor (otherwise known as the site of action), and the drug concentrations time course within the body (Birkett, 2002). Stringer (2001) relays pharmacokinetics as "the mathematical description of the rate and extent of uptake, distribution, and elimination of drugs in the body".

The pharmacokinetics and bioavailability of a drug can be affected by several parameters including fish species, age, water temperature, salinity, and route of administration (Haug and Hals, 2000). Consequently knowledge of the pharmacokinetics and bioavailability of the drug used in the intended species is vital for correct application (Samuelsen and Ervik, 1999; Stringer, 2001; Birkett, 2002; Hansen *et al.*, 2003). Favourable pharmacokinetic properties include good absorption after administration, high bioavailability, good tissue penetration and biotransformation (Stringer, 2001; Birkett, 2002; della Rocca *et al.*, 2004). Although studies have been conducted on uptake, distribution and excretion in rodents a comparison with the results from rodents show that the toxicity of

antiparasitic drugs to aquatic organisms is likely not well correlated to mammals (Yoshimura and Endoh, 2005).

Neoparamoeba perurans has been confirmed a cosmopolitan protozoan parasite and, therefore, of significance to the global mariculture industry (Young *et al.*, 2008). Thus, confirmation of *N. perurans* in regions of significant finfish production indicates that AGD is of global significance to the mariculture industry and therefore the development of a cheaper alternative treatment of this disease has the potential to become a worldwide issue as opposed to a localised Tasmanian issue.

6.1 Conclusion

This thesis evaluated the *in vitro* toxicity of bithionol and bithionol sulphoxide to *Neoparamoeba* spp. and determined that both bithionol and bithionol sulphoxide were toxic to *Neoparamoeba* spp. at concentrations ranging from 0.1 to 10 mg L^{-1} over 72 h; however, freshwater still remained the most toxic with complete mortality seen at 48 h. Following this, the toxicity of bithionol to Atlantic salmon and rainbow trout in fresh and seawater and the efficacy of bithionol as a 1 h seawater bath treatment for AGD were examined. It was determined that bithionol was toxic at and above 25 mg L^{-1} in freshwater and seawater for both Atlantic salmon and rainbow trout; whilst concentrations of 1, 5 and 10 mg L^{-1} produced an efficacy comparable to the currently used freshwater bath in Atlantic salmon. To follow on from Stage I to Stage II of drug testing, the efficacy of bithionol as an oral treatment for Atlantic salmon affected by AGD was examined. It was reported

that bithionol at 25 mg kg^{-1} feed, when fed as a two week prophylactic treatment for *Neoparamoeba* spp. exposure, delayed and reduced the intensity of AGD pathology. In order to enhance the knowledge of bithionol as an oral treatment for AGD the effects of prophylactic and therapeutic treatments were examined as well as the combination with a freshwater bath. It was identified that there was no difference in gross gill score or percent lesioned gill filaments when Atlantic salmon were fed bithionol at 25 mg kg^{-1} feed prophylactically or therapeutically. When a freshwater bath was administered there was a proportional knock down suggesting that it may be advantageous to use bithionol as a combination therapy with the current freshwater mitigation to achieve the best results for reduction in clinical signs of AGD.

The results of this thesis indicate that bithionol warrants further investigation as a possible in-feed treatment for AGD in Atlantic salmon, especially as a combination therapy with the standard freshwater bath treatment. Also, examining the effect of pulse feeding or conducting trials under more realistic field conditions with lower exposure doses and freshwater baths would be of interest. It would also be advantageous and necessary in order to develop the treatment further to examine the environmental effects along with the pharmacokinetics of bithionol.

CHAPTER 7

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8 Appendix I

8.1 Trypsin-EDTA solution in Hanks Balanced Salts Contents and concentrations

Trypsin 0.5 g L^{-1}

Ethylenediaminetetraacetic acid (EDTA) solution 0.2 g L^{-1}

Hanks Balance Salts (HBSS) Modified containing:

KCl 0.4 g L^{-1}

KH_2PO_4 (anhyd) 0.06 g L^{-1}

$NaHCO_3$ 0.35 g L^{-1}

NaCl 8.0 g L^{-1}

Na_2HPO_4 (anhydrous) 0.04788 g L^{-1}

D-Glucose 1.0 g L^{-1}

Phenol Red•Na 0.011 g L^{-1}